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The development of viral capture, concentration and molecular detection method for norovirus in foods to establish the risk to public health

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Glossary of abbreviations

+ssRNA	Positive sense single stranded RNA
ACMSF	Advisory committee on the Microbiological Safety of Food
bp	Base pairs
cDNA	complementary DNA
CPE	Cytopathic effect
Ct	Cycle threshold
dNTPs	Deoxynucleotide
dpi	Days post inoculation
dsDNA	Double stranded DNA
EC	European Commission
ECDC	European Centre for Disease Prevention
EFSA	European Food Safety Authority
EU	European Union
FSA	Food Standards Agency
G	Genogroup
g	grams
gc	genome copies
GP	General practitioner
h	hours
HAV	hepatitis A virus
HBGA	Histo-blood group antigens
HEV	hepatitis E virus
IID	Intestinal infectious disease
LOD	Limit of detection
$MgCl_2$	Magnesium chloride
mw	molecular weight
OCL	Official Control Laboratory
PEG	Polyethylene glycol
pfu	Plaque forming units
PGM	Porcine gastric mucin
PHE	Public Health England
RTE	Ready to eat foods
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-PCR units	Reverse transcriptase polymerase chain reaction units
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction units
SOP	Standard operating procedure
TBE	Tris-borate EDTA
TE	Tris-EDTA
VLPs	Virus like particles
w/v	Weight by volume
WHO	World Health Organisation

Abstract

Norovirus has been identified as a common cause of gastroenteritis worldwide, and food as a transmission vehicle has been well documented. Standardised detection methods exist for the detection of norovirus from fresh produce and molluscan bivalves, whilst detection methods for a wider range of food matrices that may be implicated in transmission of norovirus do not currently exist. The detection of norovirus in foods suspected to be implicated in transmission is paramount for appropriate outbreak investigation. The contamination of foods other than shellfish and fresh produce often occurs via food handlers. The proportion of norovirus that is typically transferred from food handlers to food also remains unknown. Understanding this is necessary in order to estimate the risk of infection and the burden of gastroenteritis caused by norovirus that is attributable to food contaminated by food handlers. These questions were addressed by the development of a combined capture, concentration and quantitative detection protocol with the aim to enhanced norovirus recovery from a range of food types.

A food surface wash and norovirus capture method that was sensitive, reduced processing time, and increased throughput capacity was applied to a range of ready to eat foods. An automated nucleic acid extraction method which further reduced processing time and increased throughput was validated. Finally the validated method demonstrated that two real time RT-PCR assays currently used for the detection of norovirus in shellfish and fresh produce or in faecal samples were comparable overall, and hence either could be used in combination with the norovirus capture, concentration and extraction protocol described in this thesis. The protocol was applied to a range of food matrices and resulted in <1% to 55% recovery of norovirus GI and <1% to 25% recovery of norovirus GII.

The optimised protocol was then used to quantify virus transfer between food handlers hands and to food, in simulation experiments where food handlers' gloved hands were artificially contaminated prior to preparation of a sandwich. This enabled norovirus transfer to food items and to other food handlers to be measured at each stage. Quantitative data demonstrated that 5.9 ± (SD ± 0.1) $\log_{10}$ cDNA copies/µl of norovirus GII inoculum, resulted in a percentage recovery of between 3.0% and 0.02% from Food Handlers and 7.8 ± (SD ± 0.1) log10 cDNA copies/µl of norovirus GI inoculum resulted in a percentage recovery between 9.6% and 0.004% from Food Handlers. The average percentage recovered from sandwich pieces over six replicates was 0.2% for norovirus GII and 1.2% for norovirus GI. The method and protocols developed could be rolled out to official control laboratories and aid foodborne outbreak investigation by allowing testing of food categories that currently are not investigated. Furthermore, this work demonstrated the extent of norovirus transfer from hands to food ingredients and the environment and could be used in risk assessment models. Further work applying these protocols to quantify the transfer from contaminated hands using a range of viral loads will be useful in determining risk more accurately, and to monitor and investigate food premises by introducing this as an additional food and hand hygiene marker.

1. Introduction

In the UK estimates of all causes of infectious intestinal disease (IID) are 17 million cases annually from a total population of 65 million people (Tam *et al.*, 2012). One of the main challenges of determining accurate estimates of all causes of IID is due to underreporting to national surveillance systems; therefore the number of cases reported in national surveillance systems is analogous to the reporting pyramid (Figure 1). Only a small proportion of the number of cases in the community present to a General Practitioner (GP), and an even smaller proportion of these sample submissions are then reported to national surveillance. In the UK IID rates reported to primary healthcare are 17.7 cases per 1,000 person years. By comparison, IID rates reported to primary healthcare in Germany were estimated as 40.2 cases per 1,000 person-years (Karsten *et al.*, 2009), and in the Netherlands 7.9 cases per 1,000 person years (De Wit *et al.,* 2001). Reporting IID to a GP is the first stage of the reporting process, and reported cases are greater in the UK than the Netherlands but less in the UK than Germany. However, the proportion of cases reported to national surveillance systems is poor globally.

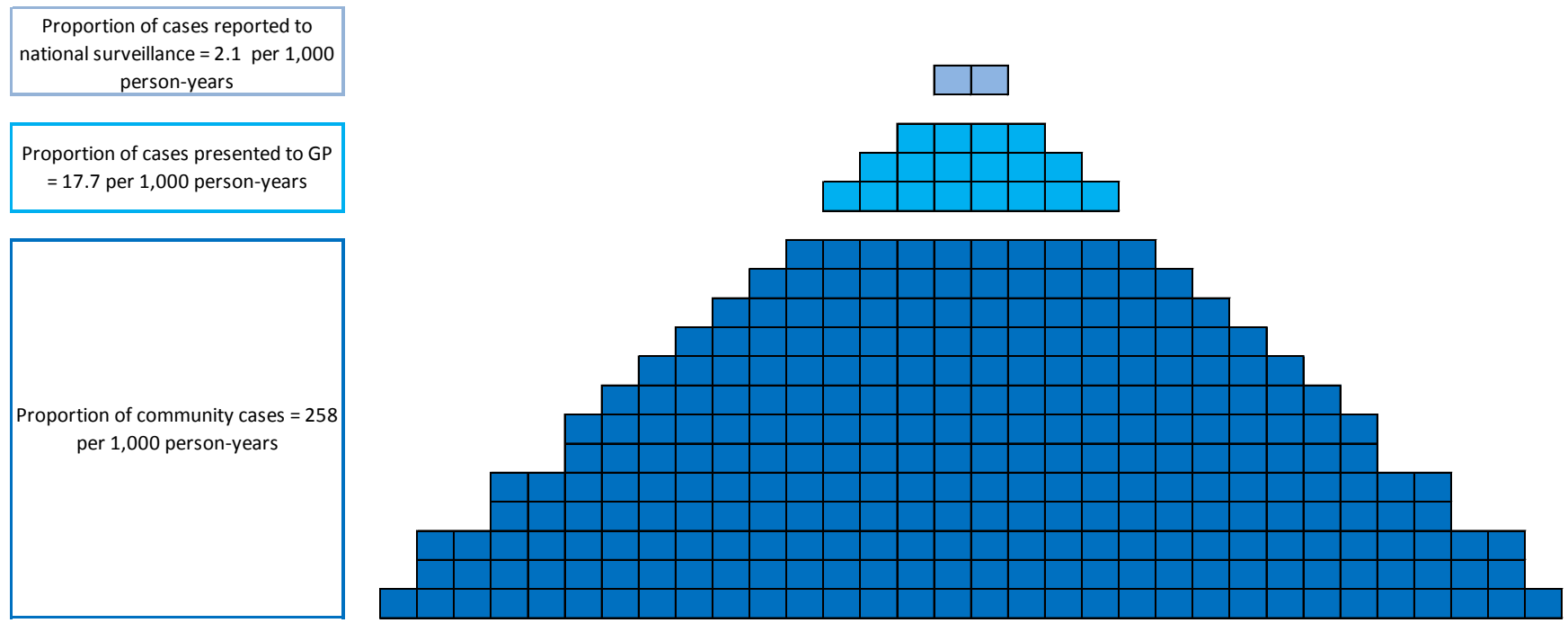


Figure 1 Reporting pyramid representing the estimated crude incidence rate in the UK and showing the proportion of community cases that are reported to a GP, and the proportion of GP cases that are then reported to national surveillance of overall IID (Tam *et al.*, 2012).

There are many socio-economic factors which are driving poor reporting to national surveillance. At each stage of the reporting pyramid cases of gastroenteritis are dependent on patients consulting primary care, patients submitting a faecal specimen so a microbiological diagnosis can be made, and microbiological laboratories sending specimens to national reference laboratories, so that these cases can be recorded in national surveillance systems. Figures on patients refraining from submitting a faecal sample have been observed in a smaller study conducted in Ireland, 29.2% of the community reported IID to their GP, but only 2.0% of patients submitted a faecal sample for testing (Scallan *et al.,* 2004). Some of the factors which deter patients from submitting a faecal sample include; symptom severity, fear of results, embarrassment, and a lack of guidance from a medical professional on how to submit a faecal sample (Lecky *et al*., 2014). Some factors which result in the failure of a laboratory diagnosis being made can be due to a lack of testing capability, no local, regional or national financial support to carry out testing, or a lack of engagement from the healthcare sector in collecting specimens for laboratory diagnosis. A lack of financial support in part may be due to the absence of a statutory requirement to test samples for certain organisms and provide comprehensive surveillance. Other reasons such as not enough staff to dedicate time to sending samples, but also due to the failure of GPs to submit faecal samples in line with national guidance has been found to contribute to the reduction in faecal specimens received by national reference laboratories (McNulty *et al*., 2014). Currently all of these factors are contributing to a lack of visibility in the number of cases of IID that occur in the community, making it difficult to link cases and causes of IID to outbreaks and to identify other potential transmission routes such as through food or food handlers. By not having this information it is difficult to demonstrate the impact this has on public health, making it difficult to identify failures in food safety or practises.

Global estimates made in the Foodborne Disease Burden Epidemiology Reference Group (FERG) report 2015 state that 29% (95% CI ±6%) of cases of disease are caused by diarrheal pathogens, and 582 million cases (95% CI 401–922 million) were transmitted by contaminated food, resulting in 25.2 million (95% CI 17.5–37.0 million) disability adjusted life years (DALYs). Globally, estimates of viral illness associated with transmission via food are in the range of around 5% for hepatitis A, 12-47% for norovirus and are unknown for hepatitis E (WHO, 2008). Although it is acknowledged other forms of disease caused by food associated viral pathogens, such as hepatitis A and hepatitis E include liver disease and death, the best estimates of the burden of foodborne disease are available based on gastroenteritis caused in individuals who acquire foodborne disease. However, under-reporting to national surveillance systems means there is a lack of epidemiological data, resulting in an underestimation of cases (O'Brien, 2008). This makes it difficult to determine the accuracy of UK and global estimates of viral illness attributed to food.

1.1. Outbreak reporting surveillance and investigation

Outbreaks of disease and foods that fails to comply with current regulation or guidance are notified to the Food Standards Agency (FSA) and local official control laboratories (OCLs) may become involved in the testing of the implicated food. Food will be tested by OCLs when the food is at production or on the market at retail sale, and any issue with the product may mean that it is defined as unfit for human consumption or injurious to health (European Commission 2004). This can occur for a range of issues, including that the food contains prohibited or undeclared ingredients, chemicals or drug residues, or that it contains microbiological pathogens, such as those defined in Regulation (EC) No. 2073/2005 (as amended). In accordance with Regulation (EC) No. 851/2004, ECDC operates a technical platform, the Epidemic Intelligence Information System for Food and

Waterborne Diseases (EPIS-FWD). This platform is designed for public health experts to exchange information to be used in the assessment of any unusual increase in cases, and the sharing of molecular typing data to link cases across countries, where food or water is involved in transmission. As standard methodologies do not exist for all food matrices it makes source attribution difficult to achieve, so the burden of food as a transmission vehicle is not well understood. Furthermore the EPIS-FWD information sharing platform is currently not joined up to the rapid alert system for food and feed (RASFF), which was launched by the European Commission to monitor food safety. The Food Standards Agency (FSA) is the only UK Government Agency to input surveillance data onto the RASFF system. In total there were 2839 entries made onto the system in 2015, of which 287 entries were made by the FSA for the UK. In 2016 the total number of entries was 3046, of which 365 entries were made by the FSA for the UK. Although this is approximately a 12% data contribution, none of these reports relate to viruses in foods. In total, only 23 entries of viruses in foods were reported onto the RASFF system in 2016, and 20 entries in 2015 were made by EU member states. In the latest published EU summary report on trends and sources of zoonoses, zoonotic agents and food associated outbreaks this equated to 0.09 reported cases per 100,000 population (EFSA, 2016). To move this area of epidemiology forward, it will be important that laboratory and epidemiological data are combined in future surveillance systems. Until this occurs, the consequence is that there is no official virus testing and reporting service offered by the Public Health England Food, Water and Environmental Microbiology Service (FWEMS), which includes the PHE Official Control Laboratories in England. Existing legislation relies upon testing and reporting of all food and for bacterial coliforms; faecal indicators of food spoilage for the purpose of food safety, surveillance or outbreak investigation (EU Directive 80/778/EEC & EU Directive 91/492/EEC). The use of bacteria as an indicator of faecal contamination and virus

contamination has been criticised in the literature as inaccurate and not fit for purpose by many studies (Lowther *et al.,* 2012, Mesquita *et al.,* 2011, Suffredini *et al.,* 2008 Croci *et al.,* 2007).

Enteric viruses are a principle cause of food associated disease. The European Food Safety Authority (EFSA) have reported and highlighted the three key viruses causing food associated disease are hepatitis A, hepatitis E and norovirus (EFSA, 2013). These three viruses have been associated with sporadic cases and outbreaks of disease where transmission of the virus has been associated with faecal contamination in the food chain; either originating at source, entering the food chain from environmental reservoirs, or as a consequence of handling by food handlers excreting the virus and failing to practice good hand hygiene (Figure 2).

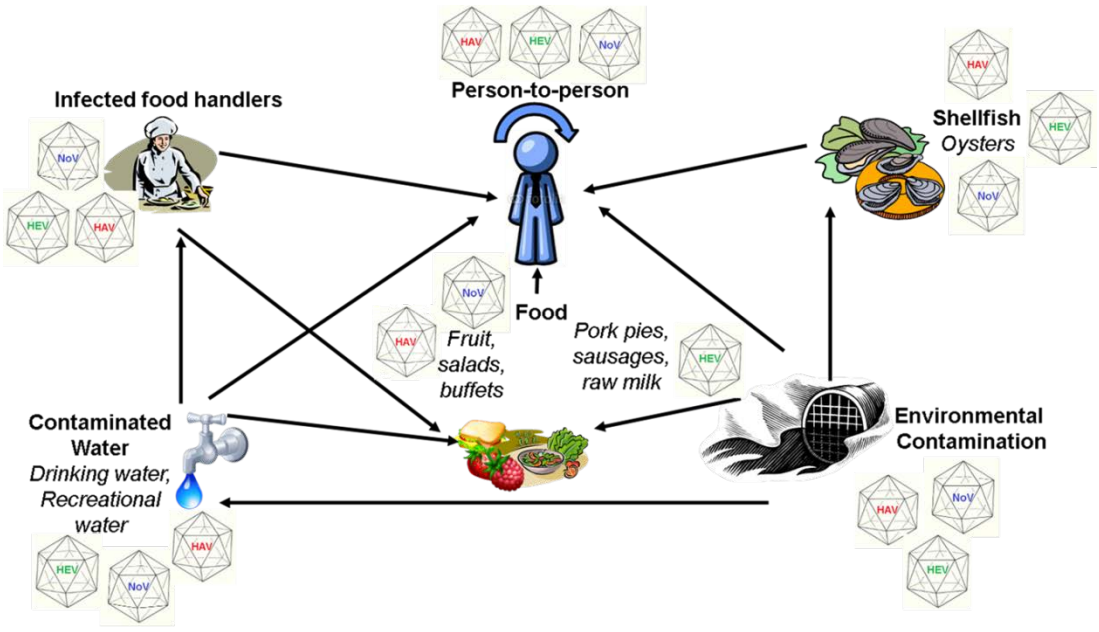


Figure 2 A diagram showing the common transmission routes of norovirus, hepatitis A and hepatitis E between food, water, the environment and infected individuals (adapted from Allen *et al.*, 2013).

The European Centre for Disease Prevention and Control (ECDC) recognises that a more focused response in this area is needed, and in section 10.3 of the ECDC strategic programme, proposes to strengthen data collection and integration between epidemiological and virological data, for hepatitis A virus and norovirus in food and water samples (ECDC, 2014). Combining epidemiological and sequencing data has allowed the linking of foods to outbreaks in some studies (Boxman *et al.,* 2011, Müller *et al.*, 2016, Made *et al.*, 2013). Therefore, this is a step forward in improving European surveillance of food associated viruses; however this strategy does not mention the incorporation of hepatitis E virus, which has been identified as a significant emerging zoonoses, with laboratory confirmed cases in the UK increasing year upon year since 2010 (Said *et al.,* 2014)

1.1.1. Hepatitis A virus

Hepatitis A belongs to the *Picornaviridae* family of positive sense single stranded RNA viruses. The virus has a genome of 7.5 kilobases and is transmitted via contaminated blood products or through the faecal-oral route, through person-to-person contact and/or consumption of faecal contaminated food or water. Food may become contaminated with faeces containing hepatitis A during growth, harvesting, production or preparation by infected food handlers. Three genotypes infect humans and these are organised as seven subtypes. Despite the genetic diversity of hepatitis A, all genotypes are part of a single serotype, and this characteristic was exploited in vaccine design and implementation, resulting in lifelong immunity (Robertson *et al.,* 1992). The vaccine is only provided to high risk groups such as men who have sex with men or healthcare workers. It is also provided in travel clinics for people visiting endemic countries (PHE, 2013), where sanitation is poor and transmission is high such as Eastern Europe, Africa and Asia. However control

measures such as good hand hygiene and vaccination of individuals at high risk of exposure, have been shown as effective control measures (Klevens *et al.,* 2010). Hepatitis A tends to be self-limiting with clinical symptoms including gastroenteritis, myalgia, fever and malaise. It is rarely fatal in healthy individuals; however it can lead to more severe clinical illness such as jaundice and hepatomegaly, particularly in older individuals. In comparison 70% of younger individuals under six years old are asymptomatic making infected individuals difficult to identify (Hadler *et al*., 1980). Incubation periods can last between 15-50 days before onset of clinical symptoms. This combined with cases of overseas travel, can make it difficult to recall consumed food items, and retrieve foods for testing, making it difficult to identify any suspected food as the cause of illness. Consequently it has been estimated that 48% of patients with hepatitis A infections do not identify a source (Fiore *et al.,* 2006). Although there are substantial differences in healthcare systems in the USA compared to the UK, the economic burden of hepatitis A has been estimated there at a cost of $488.8 million annually for the treatment of adolescents and adults (Berge *et al*., 2000). The cost per outbreak has also been reported between $3,824 to $200,480 per case, with post exposure prophylaxis being a major contributing cost (Luyten and Beutels, 2009).

Examples of international food associated outbreaks have been described. Based on a review by Sharapov *et al*. (2016) transmission via an infected food handler may be a common route of transmission. In this review 192 hepatitis A infected food handlers were identified to have worked while infectious, and 18% of the infected food handlers were reported to have transmitted to restaurant diners. The number of restaurant diners as secondary cases is not known from this review. However, the 192 hepatitis A infected food handlers were confirmed by epidemiological data on databases of local and state health

departments in America over a 19 year period. Overall food associated outbreaks of hepatitis A are rare in countries where the virus is endemic as immunity in the population is high. However, food associated transmission to non-immune travellers to endemic regions may be a common route of food associated transmission (Wheeler *et al.,* 2005). A study by Klevens *et al*. (2010) identified over a two year period that from 1156 cases of hepatitis A overseas travel to endemic regions was reported in 46% of these cases, suggesting this to be a high risk factor. All of these 1156 cases were confirmed by molecular testing and characterisation of patient faecal specimens, which is useful in identifying related cases in geographically clustered outbreaks or outbreaks of sporadic strains, however for routine surveillance purposes molecular characterisation does not provide any information on transmission routes.

Food that has been processed by asymptomatic food handlers and distributed to non-endemic countries is another transmission route. An example where this may take place has been described in the berries supply chain, in which asymptomatic fruit pickers in endemic regions of Europe who have poor hand hygiene and bare hand contact during fruit picking can cause faecal contamination via hand contact with the fruit, which is then distributed to non-endemic countries. An example of this occurred in an outbreak reported in Italy, and later in three people from Ireland who had no travel history to Italy. Epidemiological data implicated mixed berries or cakes containing mixed berries as the common food source consumed. Although mixed berry testing was increased after a European alert to the RASFF system was made, it is not known how many berry samples were tested, however hepatitis A RNA was identified in two batches of berries tested. Characterisation was achieved in the patient samples which was later identified as the same in all patient specimens (Rizzo *et al.*, 2013). No characterization was achieved in the berries.

Contamination of fresh produce and Ready to Eat (RTE) food with hepatitis A was identified due to contact with contaminated irrigation water during production, in a recent study in 2.6% (24/911) of RTE salad vegetables tested. EU Reg. 1441/2007 sets out a criteria for bacterial pathogens found in pre-cut fruits and vegetables (RTE) during shelf life, but there is no microbiological criteria for viruses (Terio *et al.,* 2017). Another international outbreak included sun dried tomatoes which were distributed globally. An association with the consumption of sundried tomatoes and illness was identified, odds ratio [OR], 3.0; 95% CI 1.4–6.7. Hepatitis A RNA was also detected in 22 sundried tomatoes and hepatitis A genotype IB was identified in 94% of patients out of a total of 153 patients tested (Donnan *et al.*, 2012).

1.1.2. Hepatitis E virus

Hepatitis E belongs to the *Hepeviridae* family of positive sense single stranded RNA viruses. The virus has a genome of 7.2 kilobases and hepatitis E can also be transmitted via contaminated blood products (Boxall *et al.,* 2006), by the faecal-oral route, through person-to-person contact and/or consumption of contaminated food or water via an infected food handler. The virus has four recognised genotypes, with genotype I and II only affecting humans and genotypes III and IV primarily infecting pigs but capable of causing human infection. A vaccine for hepatitis E genotype I has been trialled and licensed in China; but it is not yet available worldwide, therefore data regarding cross protection against zoonotic genotypes is limited (Shrestha *et al.,* 2007). Geographically hepatitis E virus is the main cause of viral hepatitis in endemic areas such as Africa and Asia where sanitation is poor, zoonotic hepatitis E outbreaks occur in developing and developed countries (Shimakawa *et al.,* 2016). Clinical symptoms differ between the zoonotic and non-zoonotic strains of

Hepatitis E. Genotypes I and II are more likely to cause symptomatic disease, including gastroenteritis, myalgia, fever, malaise, jaundice and hepatomegaly. Zoonotic disease is often asymptomatic making it difficult to identify infected individuals, although exact estimates on those who are asymptomatic are unknown. Although zoonotic hepatitis E can lead to severe illness in immune suppressed patients and may cause chronic liver disease or in rare cases may be fatal, particularly to pregnant women. Incubation periods for this virus can last between 14-40 days, also making identification of food as the source of the outbreak difficult. The financial burden caused by Hepatitis E is unknown. However, an enhanced national surveillance programme for the incidence of hepatitis E in people has only been conducted since 2003 in England and Wales, and the number of laboratory confirmed cases has increased significantly year on year from 368 cases in 2010 compared to 1244 cases reported in 2016 (PHE, 2017). Although no national surveillance of processed pork products in the UK is currently required, it is believed that processed pork is a likely source of transmission in developed countries across Europe, based on a study which found anti-HEV antibody in 85.5% of 256 pig sera tested from UK pigs (Banks *et al.,* 2004). Furthermore, a strong association with the consumption of undercooked game meat has been identified with hepatitis E (Legrand-Abravanel *et al.,* 2010). Hepatitis E virus has been found as indigenous in pigs and therefore in many processed pork products (Said *et al*., 2014) and shellfish (Mesquita *et al.*, 2016, Crossan *et al.,* 2012). An outbreak of hepatitis E virus occurred on a cruise ship and was statistically associated with consumption of shellfish onboard (Said *et al.*, 2009). There is no methodology available for testing hepatitis E from any food matrices, and is a concern as hepatitis E is an emerging zoonosis in the UK, with the majority of cases believed to occur through the dietary route (Said *et al.,* 2014).

1.1.3. Norovirus

Norovirus belong to the *Caliciviridae* family of positive sense single stranded RNA viruses, and are non-enveloped, single stranded RNA positive sense genomes contained in an icosahedral virus capsid. It is transmitted via the faecal-oral route, through person-to-person contact and/ or contaminated food or water. Food and water may become contaminated during production or through infected food handlers. The classification system of norovirus is based on complete amino acid sequences of the capsid proteins (Zheng *et al.,* 2006). This classification system was able to accurately identify the diversity amongst norovirus strains and classify them into the six genogroups (GI-GVI) with a seventh proposed (Tse *et al.,* 2012, Vinjé, 2015), of which the majority of human norovirus strains were then classified into either nine genotypes associated in GI or 22 genotypes in GII. Genogroups GI and GII are responsible for the majority of human infections, and transmissions of these have been implicated in food, therefore these genogroups will be investigated further. There are no licenced vaccines, but a bivalent vaccine has been developed and is undergoing clinical trials (Atmar *et al.,* 2015). This virus causes gastroenteritis which characteristically presents itself 24 to 48 hours after infection. Geographically norovirus has been identified as the most common cause of acute gastroenteritis worldwide (EFSA, 2013, Kirk *et al.,* 2015). Estimates on food associated IID in the UK caused by norovirus in healthy people in the community has been estimated as 36.7 cases per 1,000 person years and 142.6 cases per 1,000 person years in children under five years old (O'Brien *et al*., 2016). Estimates on norovirus prevalence and the economic impact on the UK health service and society has been estimated to cost £69 million, greater than *Campylobacter* species and Rotavirus combined (Tam and O'Brien, 2016). The recent figures on the economic impacts of norovirus alone, suggest better surveillance of all viruses is

required in order to identify the most common sources of transmission, and to implement prevention measures.

In the UK the IID2 study estimated that norovirus was the most common cause of IID resulting in 3 million cases annually based on a quantification cycle (Cq) cut off of 30 (Tam *et al.,* 2012), however recently these estimates were revised using a clinically relevant Cq cut off of 40, resulting in 3.7 million norovirus infections occurring annually (Harris *et al.,* 2017). This has been the threshold set for all norovirus positive clinical specimens, and is in agreement with MIQE guidelines (Bustin *et al.,* 2009). However, from these figures it is difficult to determine what proportion of these infections is food associated in the UK. Studies from the Foodborne Viruses in Europe network (FBVE) estimated that out of 5,036 norovirus outbreaks reported with a known mode of transmission, 10% were associated with contaminated food (Kroneman *et al*., 2008).

In the absence of a routinely available cell culture model most data is based on RT-PCR data either qualitatively identifying norovirus RNA determined by a band of the correct size by gel electrophoresis, or quantitatively by a cycle threshold (Ct) cut off at 40 (Bustin *et al.,* 2009) followed by sequence data. Few authors have successfully obtained sequence data to link contaminated food handlers, food and the environment during outbreak investigations, which may be due to the sensitivity constraints of commonly used RT-PCR assays which have been developed with clinical specimens in mind (Maunula *et al.*, 2009, Made *et al.*, 2013). Outbreaks of norovirus GII have been reported in 11,000 individuals in Germany following importation of 44 tons of strawberries from China. Norovirus RNA was successfully detected in 7/11 strawberries tested and only one strawberry provided genotyping data (Made *et al.*, 2013). Another outbreak was reported to affect 200 people in Finland in which 20,000 Kg of raspberries were implicated. Norovirus RNA was identified in

three 25g specimens, in which one specimen had a high enough viral load that it could be genotyped and was linked to the genotype isolated from patient specimens (Maunula *et al.,* 2009). A single large scale study on norovirus prevalence in food has been conducted across countries. During this study, 867 samples of leafy greens, 180 samples of fresh soft red fruits and 57 salad vegetables were tested across Canada, Belgium and France (Baert *et al.,* 2011). However, limitations in the methodology meant few food samples were confirmed by sequencing. Only 35% of the 850 specimens from which norovirus RNA was detected, were sequenced. Overall these studies demonstrate the large volume of food potentially implicated in outbreaks and the difficulty in obtaining sequence data from food. Overlap in transmission routes can also make it difficult to determine the source of an outbreak. It is important to consider the many different routes of transmission in order to implement measures and guidelines to mitigate infection.

In this thesis food associated viruses are considered with examples directed towards the investigation of norovirus only, as norovirus is acknowledged as the most common cause of acute gastroenteritis worldwide.

1.2. The role of food handlers in virus transmission

Food handlers have been implicated in the transmission of norovirus during food production (Todd *et al.,* 2007). The are many different stages of food production in which food handlers are involved, including harvest, processing, packaging, distribution, at the point of retail sale or at home. In outbreaks linked to food handler involvement, the most frequently reported risk factors are handling of food through bare hand contact, combined with poor hand hygiene (Todd *et al.,* 2007, Barrabeig *et al.,* 2010, Hall, 2012).

In catering or food production industries, there are risk management protocols that are designed to limit contamination. Businesses are required to implement a written food-safety management system, based on hazard analysis and critical control point (HACCP) principles, and to ensure food handlers are trained or instructed in good hygiene practices in the workplace (HSE, 2013). This may become disrupted due to failures in following procedure or noncompliance by staff. For example, possible reasons for food handler contamination could be due to a lack of understanding of good hand hygiene practices, due to a lack of training. This was evident in one questionnaire given to 314 participants, in which 38% of food handler employees thought it acceptable for food handlers ill with norovirus to handle packaging, food utensils and equipment, and less than half of respondents knew the appropriate cleaning solution to use when washing hands (Kosa *et al.,* 2014). Using inadequate cleaning solutions to clean hands or a lack of hand washing facilities in staff toilets has also been highlighted as a fundamental problem in infection control (Todd *et al.,* 2007, FSA, 2017). Therefore, the FSA have published documents in which correct hand washing methods are available to staff and employers (FSA, 2013). Symptomatic individuals infected with norovirus can shed high viral loads in their faeces and vomit, therefore poor hand hygiene will increase the likelihood of virus being deposited or transferred from hands to food or the environment (Teunis *et al.,* 2008, Bower *et al.,* 2000). In order to reduce this risk, the FSA fitness to work regulatory guidance and best practice advice for food and business operators (2009), states food handlers should remain off work until they are non-infectious. This has been suggested as 48 hours after symptoms have ceased (WHO, 2008), however for some individuals virus shedding can extend for prolonged amounts of time, and at high viral loads either side of the symptomatic phase (Koo *et al*., 2012, Milbrath *et al*., 2013, Soucie *et al*., 1998, Skinhøj *et al*., 1981). Although after the symptomatic phase the likelihood of shedding virus into

the environment is reduced, shedding can still occur on average up to two weeks after symptoms have stopped, and for much longer in immunocompromised individuals (WHO, 2008). Therefore, the implication is that the current guidelines may not be fit for purpose and may not be protecting food consumers from food handler contaminated meals.

Examples of non-compliance to health and safety guidelines by food workers include denying illness in themselves or a close family member. This may be due to concerns over job losses or loss of earnings, as current practice results in staff remaining unpaid during periods of sickness. Pressure to attend work due to staff shortages was also identified as a potential risk factor in increasing the likelihood of staff denying illness (FSA, 2017).

The transmission of norovirus can also occur through asymptomatically infected individuals. These individuals can have an active norovirus infection and will not be identified as a risk for transmission, but they do act as carriers of infectious virus, and so need to be considered for infection control. Asymptomatic excretion of norovirus has been identified in 12% to 16% of healthy individuals (Phillips *et al*., 2010, Tam *et al*., 2012). In another study, de Wit *et al.,* (2001) estimated 5.2% of individuals are asymptomatically shedding norovirus at any one time. Some food handlers were unaware of the role of asymptomatically infected individuals in norovirus transmission (FSA, 2017). Consequently many authors have identified asymptomatic food handlers present in catering premises. Okabayashi *et al.* (2008) identified norovirus RNA in 11.9% of faecal samples from asymptomatic food handlers in a catering facility. According to Sabria *et al*. (2016), in a study of 242 faecal specimens from food handlers and healthcare workers, 59.1% of the workers were norovirus RNA positive, and more than 70% of these workers were asymptomatic based on those cases reported to the Public Health of Catalonia over a two year period.

Norovirus infections have been reported in the home environment as a consequence of caring for ill family members, such as elderly or young individuals. Daniels et al. (2000a) identified food handler involvement in a food outbreak consequently caused by a food handler caring for an ill child at home before attending work. Lo *et al*. (1994) identified that a food handler who became ill a day after preparing a salad in a hospital kitchen, had been caring for an ill young child at home the day before preparing the salads. This indicates that this is not just a problem for catering establishments and that hand hygiene is crucial in the home as well; where individuals are less likely to adopt the hygiene practices, such as wearing gloves when preparing food for family members. Infections of norovirus in children may be asymptomatic (Phillips *et al.,* 2010, WHO, 2012) and in the presence of poor hand hygiene whilst caring for children may result in cases such as those described by Lo *et al.,* (1994) and Daniels *et al.,* (2000). The role of food handlers in the home has not been investigated thoroughly. It therefore remains unknown how common transmission occurs in this setting.

Overall, attributing the source of virus to food handlers in an outbreak event is complex, and development of public health response systems and new approaches to more sensitive laboratory methods are required to address this problem. Although bare hand contact and poor hand hygiene have been implicated in transmission of viruses to food, measures of infection control have been put in place to improve hygiene practices. It is also possible that contamination of food may occur through other routes. These include contact with contaminated fomites in food preparation areas, as a secondary source of contamination by transference from an infected food handler.

1.3. The role of fomites in virus transmission

Fomites are inanimate objects that become contaminated usually by transfer of virus from infected individuals acting as a source of virus leading to infection and seeding outbreaks. Fomites have been shown to have an important role in seeding outbreak events, and much of this evidence comes from the healthcare and community sectors. It has been shown that fomites can act as an environmental reservoir that can lead to outbreaks re-establishing even after cleaning in a hospital (Morter *et al.,* 2010). There are no exact estimates on the involvement of fomites in relation to the number of food associated outbreaks that occur (Hansman *et al*., 2010), however there have been cases of environmental reservoirs implicated in outbreaks. Davies-Cole *et al*. (2008) reported an outbreak in a school in the District of Columbia in which 27 students and two staff member were ill with gastroenteritis. Two faecal sample specimens were received and were identified as norovirus positive with the same sequence identified from a swab collected from a computer keyboard.

Food associated viruses have been shown to persist in the environment even when exposed to harsh conditions for long durations of time (D'Souza *et al*., 2006, Barker *et al*., 2004, Rzeżutka and Cook, 2004). Persistence in the environment also increases the likelihood of viruses to be transferred from one fomite to another, which is of particular concern in food preparation areas in the catering environment. Transfer efficiency is highly variable and depends on many factors, including; the pressure applied (Ansari *et al.,* 1988), contact time, the virus, the texture of a contact surface (D'Souza *et al.,* 2006, Escudero *et al.,* 2012) and whether the contact surface is wet or dry (Sharps *et al.,* 2012). High risk contact surfaces in food preparation areas include fridge and freezer door handles, which have been commonly contaminated due to the high level of hand contact and pressure

applied when used (Kassa, 2001). Studies have also demonstrated the ability of viruses to survive on a wide range of contact surfaces found in catering establishments, over long periods of time. Escudero *et al.* (2012) identified the transfer of norovirus and surrogate viruses from artificially contaminated stainless steel, ceramic and formica onto lettuce and turkey in artificial contamination experiments. Inoculation of norovirus onto the stainless steel was conducted using a faecal suspension from an experimentally infected human. Norovirus concentration was monitored at different time points, at 0 minutes when the inoculum was still wet on the stainless steel surface and after approximately 30 minutes when the inoculum had dried at ambient temperature. From this data detection by reverse transcriptase quantitative PCR (RTqPCR) showed that norovirus transferred from stainless steel to lettuce more efficiently when the inoculum was still wet (0 minutes) compared to after norovirus detection from stainless steel to lettuce after a 30 minute drying time. Norovirus was very stable and a reduction of 1-2 logs in genome copy number was observed over 42 days. The reduction in norovirus concentration on stainless steel was not statistically significant. Kim *et al.* (2014) identified the survival of murine norovirus on six food-contact surfaces over 28 days. The inoculum used was a murine norovirus cell culture suspension pipetted onto the coupon of each contact surface, which was tested at different time points, including 0 minutes when the inoculum was still wet. Murine norovirus was artificially contaminated on the coupons, dried for one hour in a laminar flow hood and then tested at nine different time points, ranging from five hours to 672 hours (28 days). Detection of murine norovirus was greatest at 0 minutes when the inoculum was still wet, and reduced by 1.5 to 2 $\log_{10}$PFU/coupon over 28 days. Differences in transfer efficiency have been identified with wet inoculation and dry inoculation of gloves and fomites with contact surfaces. Sharps *et al.* (2012) identified wet inoculation of norovirus GII-4 from a faecal sample suspension transferred at a rate of 58-60% from gloved fingertips onto

stainless steel and fruits, whereas dry inoculation of norovirus GII-4 transferred at a rate of 20-70% from gloved fingertips onto stainless steel and fruits. Stals *et al*. (2013) identified much lower transfer efficiency at 38% from glove to glove contact, 5% from glove to ham contact and 8% from glove to lettuce contact, however all transfer experiments were conducted once the faecal suspension inoculum had dried on the artificially contaminated glove before transfer to the lettuce or ham. Furthermore the results of this study were obtained by swabbing food or glove surfaces rather than testing the whole food or glove sample. Although the transfer efficiency is variable amongst studies, the stability of food associated viruses in the environment means they are able to survive on multiple surface types for long periods of time, presenting a transmission risk. Environmental swabbing of contact surfaces has been used increasingly in outbreak investigations of food preparation areas. However as previously highlighted, the use of appropriate cleaning solutions could reduce the amount of virus found on fomites and reduce the amount of transmission that may occurs via this route.

1.3.1. Person to person transmission of food associated viruses

Person-to-person spread from infected individuals via the faecal-oral route is the most common mode of transmission for norovirus (Blanton *et al.*, 2006). Modelling studies based on data from human challenge experiments suggest that norovirus has a very low infectious dose, estimated to be between 10 and 100 virus particles (Teunis *et al.*, 2008, Acheson and Fiore, 2004). Identification and detection by laboratory methods of circulating norovirus strains in humans is well developed, in comparison to detection of circulating strains in food.

1.3.2. The role of food in virus transmission

Detection of viruses in foods or in instances where food items may act as a vehicle of transmission is complex. To identify a single food item as the source of infection is difficult as many different food items are consumed. There are limited methodologies available for the detection of viruses from food and those that are in place target specific food groups. To date a fully validated methodology (ISO 15216; 2017) has been developed for the detection of norovirus and hepatitis A RNA from fresh produce, shellfish food surfaces and drinking water. Fresh produce and RTE foods are commonly identified in food associated virus outbreaks, via contact with contaminated water of food handlers during production and processing. EU Reg. 1441/2007 sets out criteria for bacterial pathogens found in pre-cut fruits and vegetables (RTE) during shelf life, but there are no microbiological criteria for viruses. For some food matrices there are no methodologies described, especially for more complex composite RTE foods, which may contain a mixture of ingredients. One study which looked to identify norovirus associated with the consumption of a wedding cake was unable to directly test the cakes, due to the unavailability of a validated norovirus assay for this food matrix (Friedman *et al.,* 2005). As with all food associated gastroenteritis attributed to viral pathogens, in the absence of food testing, the exact figures remain unknown (PHE, 2014).

Cq values observed by PCR when detecting viral contamination in naturally contaminated foods may range between 35 and 40, or even higher. A lack of sensitive validated methodologies makes it difficult to answer basic questions, such as how often do foods contain viruses, and at what point of production or processing are foods most commonly contaminated. Although it is anticipated to vary depending on the food matrix and the level of processing undertaken, the availability of testing approaches across all food

matrices will provide a better insight into food safety and highlight where interventions need to be implemented.

1.4. Virus capture and concentration

Food and environmental samples that are available to test are often present as large volumes. Combined with the low viral load of virus anticipated in naturally contaminated food, the challenge is to implement methodologies that allow the size of the food specimen to be maximised, and efficiently capture and concentrate low virus quantities from it into a volume sufficiently small to be compatible with molecular methods. Therefore a virus capture and a concentration method needs to be applied (Iker *et al.*, 2013). There are many ways in which this can be done, and consequently there is no strict approach amongst authors for the use of a particular capture and concentration method. The process of detecting norovirus from different food matrices based on methodologies that have been systematically reviewed from the literature is shown in Table 1. Many of these methods have been developed by research groups and published with certain food groups in mind, making standardisation of the methods difficult (Bartsch *et al.,* 2016). Standardisation of concentration methods is required in order to ensure reliable, sensitive and accurate results are obtained (Girones *et al.,* 2010). However, due to the wide variation in the composition of food matrices, different capture and concentration methods are suited to certain food types or analytes, making standardisation of a single approach difficult to implement. The advantages and limitations of some of the methods proposed in the literature are discussed at each stage of the detection process.

Table 1 Key approaches to norovirus detection from foods including methodologies that have been systematically reviewed from the literature describing each stage of the detection process. Different concentration methods are grouped by colour: light green=ISO 15216 (2013) testing method, green=virus absorption elution methods VIRADEL); pink= magnetic bead concentration; blue= direct RNA extraction (mw=molecular weight)

Surface wash buffer	Virus capture	concentration	Nucliec acid extraction	Detection Primer and PCR	Conventional or RTqPCR	Inoculated onto food	RT-PCR units[1] or genome copies	Author
Tris-glycine Beef Extract buffer (pH 9.5)	PEG precipitation (8,000mw)	high speed centrifugation (10,000 xg) 1:1 chloroform butanol protein precipitation	Nuclisens magnetic kit	Le Guyader (2006) primers Ultrasense one-step RTqPCR	RTqPCR		10 genome copies per RT-PCR unit	ISO 15216; (2013)
Various Tris-glycine Sodium Chloride Tris-glycine sodium chloride buffer with beef extract (pH 9.5 adjusted to pH 7.4 with NaOH)	PEG precipitation (8,000mw, 10,000mw, 20,000mw)	high speed centrifugation (12,000 xg)	QIAamp viral RNA mini kit	Kojima *et al*, (2002) primers one-step RT PCR kit (QIAgen)	RTqPCR	10^7RT-PCR units/ml	10^5- or 10^3RT-PCR units/ 5g of lettuce and ham	Park et al., (2010)
Sodium hydrogen carbonate buffer in beef extracts (pH 9.5)	PEG precipitation (8,000mw)	ultracentrigugation (28,000 xg) 1:1 chloroform butanol protein precipitation	QIAamp viral RNA mini kit	Richards *et al*, (2004) and Le Guyader (1994) primers in house PCR	conventional PCR	2.81×10^7 RT-PCR units/ml	10^4RT-PCR units HAV in 90g fresh strawberries, and 10^3 RT-PCR units HAV in 60g fresh raspberries	Rzezutka *et al*., (2005) ;Rzezutka *et al*., (2006)
Various glycine NaCL ± beef extract or Trizol (pH9.5 adjusted to pH 7.4 with NaOH)	PEG precipitation (8,000mw)	high speed centifugation (10,000 xg) 1:1 chloroform butanol protein precipitation	QIAgen RNA easy mini kit	Kageyama, (2003) primers Jothikumar *et al*, (2006) PCR	conventional PCR	10^3 to 10 RT-PCR units/ 50g of raspberries	100 RT-PCR units/ 50g of raspberries	Baert *et al*, (2008)
PBS (pH 7)	PEG precipitation (8,000mw)	Cat-floc protein precipitation	QIAamp viral RNA mini kit	Vinje et al., 1996 primers one-step access RT-PCR kit (Promega)	RTqPCR	Naturally contaminated	100 RT-PCR units/1.5g digestive diverticulum (1 oyster)	Le Guyader *et al*, (2006); Le Guayder *et al*, (2003)
PBS and Freon (pH 7.4)	PEG precipitation (6,000mw)	high speed centrifugation (8,000 xg)	Trizol	Ando *et al*, 1994, Le Guyader *et al*, 1996 primers in house PCR	RTqPCR and conventional PCR	10^4, $10^3$and 10^2 RT-PCR units/ 40g ham	10-100 RT-PCR units/ 40g of ham and turkey (Equivalent to 10^2-10^3 viral genome copies)	Schwab *et al*, (2000)
Tris-glycine beef extract (pH 9.5)	PEG precipitation (6,000mw)	ultrafiltration (35,000 xg)	QIAamp viral RNA mini kit or Nucliesens magnetic kit	Häfliger, Gilgen et al., 1997 primers and Sensiscript RT kit (QIAgen)	RTqPCR	540, 54, 5.4 RT-PCR units/15g berries	54 RT-PCR units/15g of frozen raspberries	Butot, (2007)
Citrate buffer (pH4)	HBGA coated beads and iCropTheBug (Filtafelx, Pathatrix Matrix MicroScience Ltd)		QIAamp viral RNA mini kit	Kageyama, (2003) primers Jothikumar et al., (2005) PCR	RTqPCR	10^4 copies to 10/250ml buffer	green onions 10^3copies/ 250ml buffer Lettuce and Deli Ham 10^3 copies/ 250ml buffer 10^4 copies/250ml buffer	Morton *et al*, (2009)
PBS (pH 3.5)	PGM coated magnetic beads		Heat release or QIAamp viral RNA mini kit	Kageyama (2003) primers one-step RTqPCR kit (QIAgen)	RTqPCR	100,000-1 RT-PCR unit/ 140µl of PBS	1 RT-PCR unit/ 140µl of PBS	Tian *et al*, (2010); Tian et al., (2012)
Trizol (Invitrogen) (pH 4.8-4.9) and centrifugation (x8,000g)				RNeasy mini kit	RTqPCR and conventional PCR	10^3 to 10 RT-PCR units	Penne salads and frozen raspberries 10^2RT-PCR units/ per 50 g frozen raspberries	Baert *et al*, (2008)
Trizol (Invitrogen) (pH 4.8-4.9)				Häfliger, Gilgen *et al* , 1997 primers	RTqPCR	1-1,000 RT-PCR units	10 RT-PCR units/ 10g of ham	Boxman *et al* ,(2007)

[1]1 RT-PCR unit=10 genome copies

Most commonly implemented concentration methods are known as VIRADEL (virus adsorption elution) methods which were established in the 1970s and continue to be widely used in food and water microbiology (Sobsey *et al.,* 1978, Sobsey *et al.,* 1974). VIRADEL methods involve the concentration of large volumes of specimen by physical separation of the specimen through ultracentrifugation or ultrafiltration, and the immobilisation of the virus to microporous filters. These methods can be combined with precipitation techniques using organic compounds, such as Polyethylene Glycol (PEG) and chloroform. These methods are relatively inexpensive, are commonly used and are regularly cited in the literature (Rzezutka *et al*., 2005, Rzezutka *et al.*, 2006, Coudray *et al.,* 2013, Le Guyader *et al.*, 2006). The methods exploit low pH to favour electrostatic and hydrophobic interactions, which aids absorption of virus particles to solid phases. The pH is important as it can vary amongst different food matrices, and it can impact on the mechanical properties of norovirus capsids and their interactions with other molecules (Cuellar *et al.,* 2010). Variation in the pH of surface wash buffers is shown in Table 1, however wash buffers are usually in the range of pH 7 to 9.5 before being reduced to a more acidic pH to aid absorption to solid phases. It is important that the impact of pH is understood so that virus capsids are not destabilised prior to capture, particularly in cases where the capture method is dependent on virus capsids remaining intact. Extreme pH conditions can be exploited to destabilise the capsid and increase the release of the viral nucleic acid at a later stage in the detection process.

The PEG precipitation and ultracentrifugation method has been identified by some authors as a better method for virus concentration than ultrafiltration, particularly for food matrices such as berries. It has been found that filters used in the VIRADEL technique may become blocked by the sample matrix and the available binding space on solid phases is

compromised through co-concentration of inhibitors, resulting in poor virus recovery (El-Senousy *et al.,* 2013). Made *et al.* (2013) suggested that when testing raspberries by ultrafiltration, raspberry seeds were reported to block the filter pores resulting in poor concentration of the sample and poor virus recovery, in comparison to concentration by PEG and ultracentrifugation methods where this was not a risk. This was also observed in a study by Sair *et al*. (2002), in which spin columns became blocked by faecal material reducing the ability to concentrate specimens efficiently. This is one of the disadvantages of ultrafiltration and it highlights the importance of clarifying specimens prior to virus concentration so that food or faecal matrices are not carried over. A disadvantage of both concentration techniques is that they both require the use of specialist equipment, which can be expensive and results in maintenance costs, which may deter laboratories from being able to adopt these protocols. There are potential health and safety implications around the use of these pieces of equipment. The use of specialist equipment requires specific training to reduce the likelihood of accidents. However, it is advantageous that in laboratories where this equipment is already available that both these methods are relatively cost effective and easy to implement.

Alternative concentration methods include magnetic bead separation. Antibodies or non-specific ligands which capture virus particles can be conjugated to magnetic beads, and stored until ready for use. An advantage of this concentration method is that it is quick and easy to implement, and doesn't require the use of specialist equipment. Norovirus is antigenically diverse and so immune magnetic separation is not possible for the capture and concentration of different genotypes (Atmar *et al.,* 1995). Immune magnetic separation (IMS) has been used to concentrate norovirus from artificially contaminated food in one study (Park *et al.,* 2008). The author used monoclonal antibodies and IMS,

finding that the homologous virus was efficiently captured, but that virus types heterologous to the type used to generate the antibody were poorly recognised, demonstrating that IMS was not sufficiently broadly reactive to be a useful approach. This approach is therefore not fit for purpose in a public health laboratory setting. Due to the antigenic diversity of norovirus, polyclonal antibodies would need to be constantly reviewed and once developed, would not be future-proof due to the evolution of new norovirus strains (Lindesmith *et al.,* 2008). Non-specific ligands such as histo-blood group antigens (HGBA) have been identified as attachment factors in the capture of different norovirus genotypes (Cannon and Vinje, 2008, Harrington et al., 2004, Kubota et al., 2012, Marionneau et al., 2002, Tian et al., 2010, Wang and Tian, 2014). This is an advantage compared to using highly specific antibodies.

1.5. Histo-blood group antigens as attachment factors for norovirus

Histo-blood group antigens (HBGAs) are a group of glycans containing structurally similar saccharide moieties. They can be involved in many biological roles, such as energy storage cell adhesion and membrane integrity, extracellular receptors for ligands, transportation of small molecules across membranes and cell to cell signalling. However, they contain many internal and terminal recognition sites which can be exploited by micro-organisms. HBGA can be found predominantly on the intestinal mucosa and saliva secretions, they can also be found on erythrocytes depending on whether the individual has a functional *FUT2* gene, which is also known as a secretor phenotype. Approximately 80% of European and Africans have a functional *FUT 2* (secretor gene) (Harrington et al., 2004), allowing expression of H HBGA in bodily secretions. Secretor status has been linked to susceptibility to infection and expression of symptoms with certain norovirus strains, whereas individuals which do not express ABH antigens in their saliva or intestinal mucosa, have been associated with protection against norovirus binding and infection of certain strains (Lindesmith *et al.*,

2003, Donaldson *et al.*, 2008, Shirato, 2011). Figure 3 demonstrates the synthesis pathways for the main HBGAs found on the most commonly studied type 1 and type 2 chains involved in norovirus recognition for those with secretor and non-secretor status.

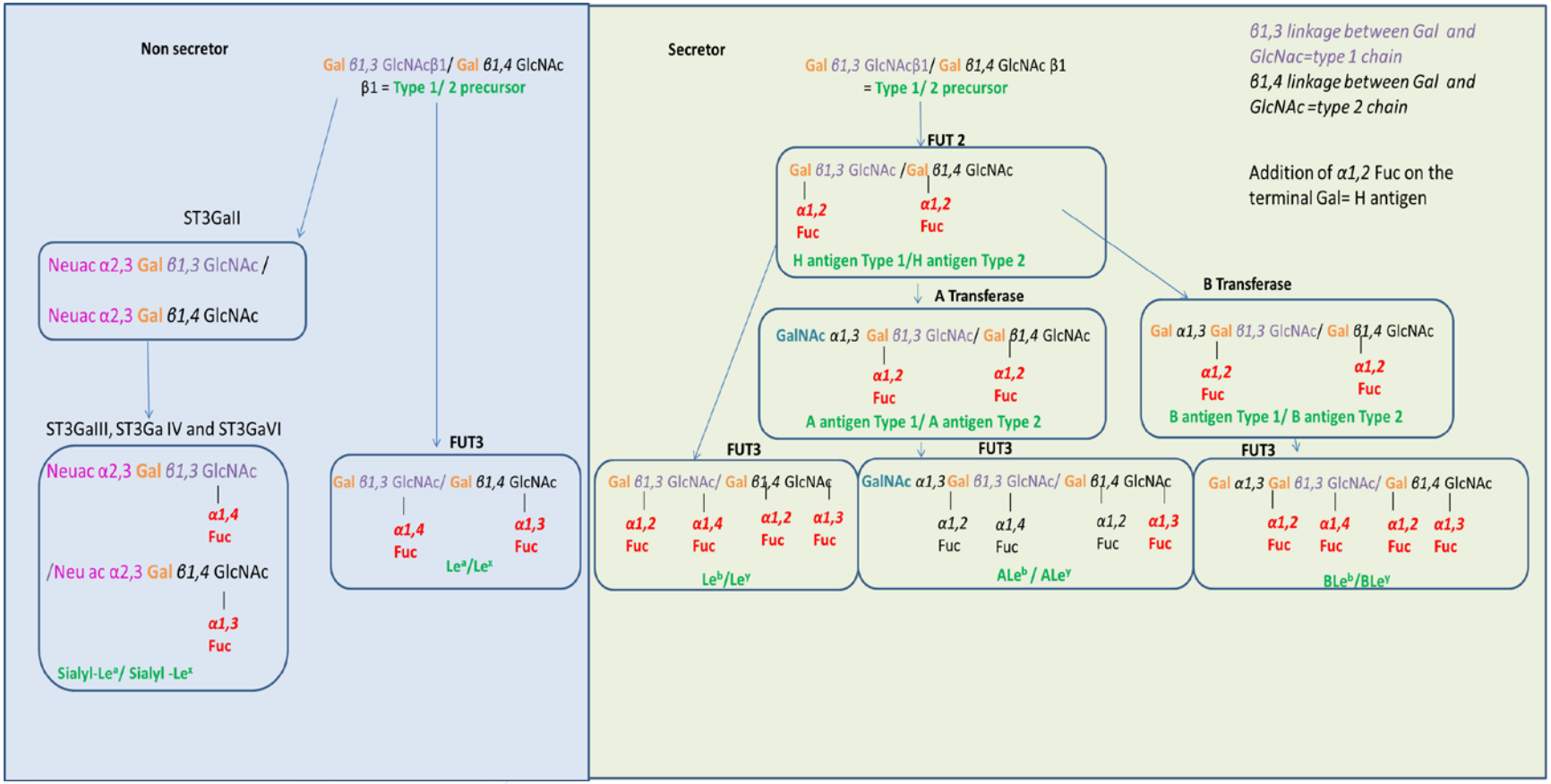


Figure 3 Adapted from de Graaf *et al*. (2016) Biosynthesis of HBGA's on type 1 chains and type 2 chains . Pale blue box identifies an example of HBGAs combinations from a non-secretor, (no A,B or H HBGAs in bodily secretions) modified by the Lewis blood group system, or sialic acid. Green box identifies HBGA combinations from a secretor of A,B or H HBGAs some of which are contained within PGM, modified by the Lewis blood group system. Type 1 (Galβ1-3GlcNAcβ1-R) and Type 2 (Galβ1-4GlcNAcβ1-R) precursor chains of both secretor and non-secretor status, modified by either FUT 2, A transferase, B transferase of the ABH blood group system and or FUT 3 of the Lewis system Red= Fuc=fucose, Orange=Gal=galactose, Blue= GalNAc= *N*-acetylgalactosamine, Purple=GlcNAc=N-acetyleglucosamine type 1 chain, Black=GlcNAc=N-acetyleglucosamine type 2 chain, Pink= Neu ac= sialic acid

Not only has norovirus binding in the presence of these HBGA been identified in humans, this has also been identified in pigs, muscles, oysters and clams (Tian et al., 2007b, Tian et al., 2007a). Porcine gastric mucin (PGM) has been identified to contain type A, type H1 and Lewis b HBGAs (Tian *et al.,* 2008). The expression of theses antigens have been identified in pig and human intestinal secretions and the presence of these antigens have been implicated in porcine and human norovirus infections (Tian et al., 2007b, Shirato, 2011). This product is a readily available commercial product and surrogate for purified human HBGA oligosaccharides. PGM has been identified to bind recombinant norovirus virus like particles (rNVLPs) and has shown to competitively inhibit rNVLPs binding to human HBGA in a dose-dependent manner (Tian *et al.*, 2005, Marionneau *et al.*, 2002). Therefore these interactions can be exploited for method development. The application of PGM to capture norovirus from complex analytes has been demonstrated from food and sewage (Tian *et al.,* 2010, Tian *et al.,* 2012). Whilst interactions between HBGA and certain norovirus strains have been identified, there may be other receptors or attachment factors required for norovirus entry into cells, particularly as it has been found not all norovirus strains interact with HBGAs (Nordgren et al., 2010). Furthermore it has been identified that in cells that overexpress HBGAs, human norovirus infection was not established. Therefore, it is proposed HBGA are a co-receptor aiding norovirus entry into cells in conjunction with a primary receptor (Le Pendu *et al.,* 2006), such as the one recently described by Orchard *et al.,* (2016) in which receptor molecules CD300lf and CD300ld enabled murine noroviruses to infect RAW264.7 cells (Orchard *et al.,* 2016, Haga *et al.,* 2016). Expression of receptor molecule CD300lf allowed murine norovirus entry into human cells HEK293T that were originally not susceptible to the virus, providing an insight into the cellular components required for virus entry.

Sialic acid has been identified as a common saccharide moiety identified by many micro-organisms. It has been suggested as an attachment factor for Murine Norovirus (DiCaprio et al., 2012). Sialic acid can be found in commercially available porcine gastric mucin (Sigma-Aldrich). Whether this compound plays a role in the capture of human norovirus remains unknown.

1.6. Extraction and purification of viral nucleic acid prior to molecular detection

Once food and environmental samples have been concentrated to an input volume suitable for nucleic acid extraction, there are a wide range of nucleic acid extraction methods available. These range from manual methods such as heat release, commercially available manual extraction kits or reagents incorporated in cartridges which can be applied to automated platforms. Alternatively, direct lysis of total nucleic acid can be achieved through chemical reagents, such as Trizol (Invitrogen). The sensitivity of these extraction methods can vary significantly, however sensitivity is an important factor when viral loads within specimens are low (Verheyen *et al*., 2012). Heat release can be applied to release nucleic acids as a cheaper alternative to chemicals; however, there are no chemical or physical measures in place to assist in the removal of inhibitors, which can affect the sensitivity of virus detection by PCR processes. This can be problematic when testing analytes such as food, as they may contain many inhibitors such as lipids, proteins, salt and preservatives. Chemical reagents such as phenol-chloroform and ethanol can be used to precipitate nucleic acids and remove PCR inhibitors. These are commonly incorporated in CE marked commercially available kits. Modern extraction methods tend to avoid the use of chloroform, due to the health and safety implications, and tend to involve lysis of virus capsids by chaotropic agents, which when disposed of appropriately is far safer. Extractions using guanidine isothyocianate, followed by capture of nucleic acid by silica or magnetic beads, and the removal of inhibitors by precipitation of proteins through ethanol is a newer

generation of extraction technology compared to the capture of nucleic acids on filters, and is incorporated in manual and automated kits alike.

Although there are a wide range of throughput capabilities by different extraction methods, automation provides the benefit of processing specimens consistently, reducing human error. This is favorable in laboratories which process a high throughput of specimens daily, as it can increase overall productivity. Some automated methods offer an on board sample tracking system to improve specimen tractability which is also favorable in a busy public health testing laboratory. Although the cost of automated extraction machines can vary significantly they can be very expensive pieces of equipment. According to Marshall and Bruggink (2006) the benefits of automated extractions has resulted in an increase in the use of these platforms routinely in diagnostic laboratories. However, few studies compare the performance of these platforms to detect target nucleic acid from specific analytes before investing financially in the technology.

The flexibility provided by the automated extraction machines make it possible to tailor protocols to specific detection needs, but this makes comparisons in the performance of methodologies between studies difficult. This is complicated further by the flexibility in sample volume that is inputted into the molecular process and is not often clarified in studies, making it difficult to compare methods.

1.7. Detection by real time RT-PCR

Once extracted, the target genome can be detected by real time RT-PCR. The real time RT-PCR process is a highly sensitive detection method and remains the gold standard for food and environmental microbiology, due to the absence of a routinely available cell culture model for norovirus (Bosch *et al.*, 2008). Although PCR is a highly sensitive technique, a limitation of the technique is that it only detects viral RNA, which is not an indicator of the

number of intact infectious particles. Furthermore, due to the low level virus contamination anticipated in foods, and the low level virus contamination required to cause norovirus infection, it can be difficult to differentiate genuine positive real time RT-PCR signals compared to background real time RT-PCR signals. Therefore, research questions still remain around the probability of infection in relation to genome copies recovered by real time RT-PCR (Baert *et al.,* 2011).

All food associated viruses discussed in this thesis are single stranded RNA viruses; therefore, target genomes can only be detected by RT-PCR or RTqPCR as one step or two step reactions. Two step PCR reactions generate randomly primed cDNA followed by PCR amplification using specific primers. In comparison a one-step real time RT-PCR reaction uses specific primers in the reverse transcription of RNA followed by specific primers in the PCR reaction. Randomly primed cDNA generated in two step real time RT-PCR reactions can be used in the analysis of different genome targets from a single preparation of the specimen. In a study by Pang *et al*. (2005) compared a one-step and two-step real time RT-PCR reaction. It was found that randomly primed cDNA was more sensitive than using gene specific primers for cDNA synthesis.

Quantitative real time RT-PCR is a useful tool in determining the amount of viral RNA in a specimen. Low viral loads of food associated viruses have been associated with causing infection. This increases the importance of having a highly sensitive PCR assay with the ability to detect low level contamination. Determining sensitivity limits of real-time assays is conducted by relative quantification of the number of genome copies in a sample based on the number of known genome copies in a plasmid used to generate a standard curve. Standard curves may be generated by a ssRNA molecule obtained after *in vitro*

transcription of cloned cDNA of the target virus, or by double stranded DNA plasmids. DNA plasmid standards are exposed to the PCR step only, therefore amplification of target virus from specimens, generated through RT-PCR may not be strictly comparable with the standards, as the standards are not included in the RT step, which has been identified as a critical step. However, a study by Costafreda *et al*. (2006) found comparable detection limits from +ssRNA molecules or dsDNA standards, in both one and two-step PCR assays.

The ability to apply characterisation techniques to link low level norovirus positive specimens obtained from food, people or the environment in outbreaks is useful. Therefore, an understanding of the norovirus genome and regions targeted during real time PCR and conventional PCR assays for characterisation purposes is explained.

1.8. Norovirus molecular biology

The norovirus genome is 7.5kb in length and is organised at three open reading frames (ORF) (Figure 4). ORF 1 encodes an approximately 200kDa polyprotein which is cleaved by the virus-encoded protease (NS6/3C like protease) into six non-structural proteins required for virus replication. These proteins in order from 5' to 3' include; p48, NTPase, p22, VPg, $3CL^{pro}$, and RNA dependant RNA polymerase (RdRp). ORF 2 encodes the major capsid protein VP1, and ORF 3 encodes the minor capsid protein VP2. The ORF1-2 junction is the target of molecular detection assays in order to distinguish between norovirus genogroups (Bull *et al.,* 2005, Bull *et al.,* 2007). The norovirus capsid is made up of 180 VP1 proteins. The VP1 has a molecular weight of approximately 60 kDa and is comprised of the shell (S) and protruding (P) domains. The P domain is subdivided into P1 and P2 subdomains. The P2 domain is hypervariable, and genotypic variations are believed to impact on HBGA binding patterns and may result in neutralising antibody escape (Lochridge *et al*., 2005, Tan *et al*., 2003). Research using VLP's has identified the P2 hypervariable domain which contains

sites that attach to carbohydrate moieties (Hutson *et al.,* 2002; Huang *et al.,* 2003, Harrington, 2004, Huang *et al.,* 2005). Based on data from crystallography studies the structural folding of the P2 domain is structurally similar across norovirus genogroups, however HBGA binding sites between GI and GII differ in location and structural characteristics (Cao *et al*., 2007, Kubota *et al*., 2012, Choi *et al*., 2008, Shanker *et al*., 2011, Shanker *et al*., 2016, Lindesmith *et al*., 2008, Tan *et al*., 2004). Although not all norovirus genotypes recognise HBGA patterns different binding patterns identified by authors for some norovirus genotypes can be found in Table 2, with norovirus GII-4 and GII-10 demonstrating the largest range of HBGA interaction (Hansman *et al.,* 2011). Emergence of new norovirus strains due to amino acid substitutions in the P2 domain result in changes to HBGA binding patterns (Shanker *et al*., 2016). Accumulation of point mutation in the norovirus genome is high due to the error-prone replication of the +ssRNA genome, particularly in the hypervariable P2 domain, leading to amino acid variations that alter the antigenic profile of the virus. Selective pressure from population immunity drives selection of antigenically different (escape mutant) strains which are capable of emerging and causing epidemic waves of gastroenteritis, and replacing other strains from circulation (Allen *et al.,* 2008, Lindesmith *et al.,* 2008).

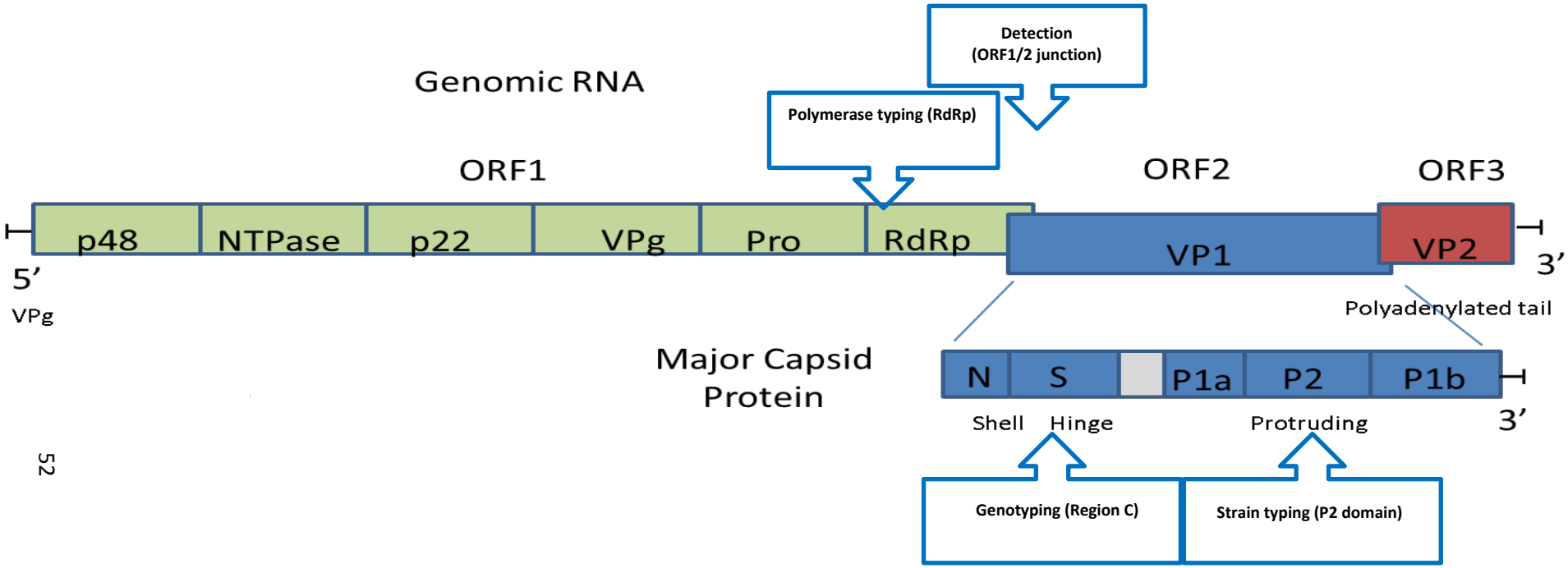


Figure 4 Diagram adapted from Donaldson *et al.,* (2008) showing the norovirus genome and demonstrating ORF 1 (Green) a polyprotein which is cleaved to make seven nonstructural proteins, ORF 2 (Blue) which contains the major capsid protein VP1 comprised of N= S=the shell domain, which is connected by a flexible hinge (H=hinge region) to the protruding domain (P domain) located at the exterior of the capsid and containing determinants of genotype specificity. The ORF 3 (Red) which contains the VP2. Parts of the genome which are targets of PCR assays described in this thesis are in blue boxes.

Table 2 A table summarising different norovirus genotypes, the accession number from GenBank, and Histo-blood group antigen (HBGA) binding patterns identified from published papers

Strain / GenBank Accession number	HBGA binding pattern	Reference
GI-I Norwalk US /M87661	A, H type 1	(Huang, Farkas *et al*. 2003, Huang, Farkas *et al*. 2005)
GI-I MOH / AF397156	A type 1, B type 1, ALe^{b}, and BLe^{b}	(Huang, Farkas *et al*. 2003, Huang, Farkas *et al*. 2005)
GI-2 / JQ743332.1	A, H3, Le^{a}	(Lindesmith, Donaldson *et al*. 2010)
GI-3 / JQ743330.1	Le^{a}	(Lindesmith, Donaldson *et al*. 2010)
GI-4 / JQ743331.1	A,$Le^{a,}$ Le^{x}	(Lindesmith, Donaldson *et al*. 2010)
GI-8 Boxer / AF538679	$Le^{b,}Le^{y}$	(Huang, Farkas *et al*. 2005)
GII-1 Hawaii / U07611	A,B, Le^{b}	(Huang, Farkas *et al*. 2005)
GII-2 Snow Mountain Virus/ AY134748	B, H3	(Tan and Jiang 2007)
GII-3 Mexico/ U22498	A,B	(Huang, Farkas *et al*. 2005)
GII-4 / VA387	ABH, Le^{b} and H type 1	(Huang, Farkas *et al*. 2003, Huang, Farkas *et al*. 2005)
GII-4 Grimsby / AJ004864	A,B,H1,H3,$Le^{b,}Le^{y}$	(Tan and Jiang 2007)
GII-4 Hunter / DQO78794	A,B,H , Le^{b}	(Shanker, Choi *et al*. 2011)
GII-10 / AF504671	A, B,H2, Le^{y}, Le^{b}	(Schroten, Hanisch *et al*. 2016)
GII-13 Parris Island / AY652979	A,B,Le^{b}	(Huang, Farkas *et al*. 2005)

1.9. Characterisation by conventional RT-PCR and dideoxynucleotide sequencing

As identified from the literature, regions of the genome encoding NS7 and VP1 (3'-end ORF1 and ORF2) are widely used in detection and characterisation of norovirus (Vinje, 2015). The ORF1/ORF2 junction is a common site of recombination; therefore characterisation assays target either side of this junction (Bull *et al.,* 2007). Early studies proposed the amplification of the polymerase (ORF1) as it was considered a conserved domain (Green *et al*., 1993 ; Ando *et al*., 1995), therefore broad primer sets were designed to amplify this region, and could be used to link outbreak cases (Vinje and Koopmans, 1996, Ando *et al.,* 1995, Wang *et al.,* 1994, Green *et al.,* 1993). However, the ORF 1 does not cluster as distinctly as the ORF 2 (Vinje *et al*., 2004). Sequencing either one of these regions in isolation against a reference sequence of known genotype is used to identify the genotype of the unknown sequence and basic phylogenetic relatedness. However, sequencing either one of these regions in isolation does not identify recombinant viruses (Bull *et al.,* 2007) or provide sufficient information to identify a transmission event (Lopman, 2006).

Once the genotype is determined, specific primers can be used in the amplification of the genes encoding the P2 domain, which is located within the ORF 2. Genotype specific primers are used to amplify the P2 domain as it is the hypervariable region and differs in size amongst genotypes. On analysis of the sequences of the hypervariable P2 domain, putative transmission events can be established, as viruses from patients where the norovirus P2 domain sequences are identical are considered to be linked in an outbreak event (Xerry *et al.,* 2008, Morter *et al.,* 2011). However, Sukhrie *et al.* (2013) proposes the duration of norovirus shedding should also be taken into consideration when using sequence data to support outbreak investigations as variation increases alongside duration of shedding. In their study they concluded minor sequence variation should be interpreted

in relation to timing of sampling since onset of illness. Minor variation consisted of no more than 2 nucleotide changes in the P2 domain, which can be observed 4 days following infection. As the P2 domain is the co-receptor binding domain (Cao *et al.,* 2007) and contains the epitope which is targeted by neutralising antibodies (Lochridge *et al*., 2005, Tan *et al*., 2003) it has been identified that changes in the P2 domain can make strains antigenically distinct (Allen *et al.,* 2009). Those mutants with increased fitness are selected from the progeny by environmental factors such as the host immune response. For immunocompromised patients virus clearance is slower than acute case of norovirus therefore the virus can replicate in the gut for longer and acquire mutations in a short time frame (Bull *et al.,* 2012). As immunocomprimised patients may shed norovirus for longer periods of time they may acquire more mutations throughout the duration of shedding resulting in complex quasispecies, from which new norovirus strains may emerge ready to infect other hosts. Whereas in immunocompetent individuals norovirus adaptation is suppressed due to a more robust immune response, resulting in development of less complex quasispecies (Karst and Baric, 2015).

Mutations outside of the ORF 2 have also been found important in identifying transmission events (Kundu *et al*., 2013). Alternative sequencing methods have been proposed including the amplification of 3,255bp comprising the polymerase-encoding sequence of ORF 1, the entire ORF 2, ORF3, and the 3’ untranslated region. The analysis of these nucleotide sequences have been used as a way to identify closely related strains in hospital outbreaks (Dingle, 2004). This approach may help to identify recombinant viruses; it may also help to discriminate between closely related norovirus strains in outbreaks that occur in a geographically small area. It may also help to address the issue of re-emerging viruses that occur due to small mutations occurring in the polymerase region and not changes in the

capsid proteins. However, amplification of 3,255bp of the norovirus genome is quite complex and for practical reasons amplification of smaller fragments, such as the P2 domain, is often conducted particularly from specimens of low viral load. Therefore, it may be more suitable to analyse the P2 domain of GII-4 sequences in outbreaks as they are the most common genotype worldwide, whilst typing information obtained from the polymerase and region C may be enough to identify food associated outbreaks in non GII-4 variants, as in contrast to GII-4, non GII-4 strains have been identified to contain one or more changes in the P2 domain over decades (Parra *et al.,* 2017). Therefore, sequence analysis of both the polymerase and capsid region could be important in identifying public health risk, although it may not be deemed cost effective.

It is important to consider that these characterisation techniques have been developed using faecal or vomit specimens, which are likely to contain high viral loads. Currently the ability to obtain complete P2 domain sequence data (445bp) from food specimens containing low viral loads so that these can be compared to faecal specimens is difficult. Baert *et al.* (2011) found that in a study of 266 norovirus RNA positive food specimens by real time PCR, only 7% were confirmed by amplicon sequencing. Made *et al.* (2013) was successful in genotyping norovirus recovered from one strawberry specimen in the outbreak of which 44 tons of strawberries were implicated. Maunula *et al.* (2009) also reported detecting norovirus by real time RT-PCR in three out of five raspberry samples, and only successfully sequencing from one frozen raspberry sample which had a high viral load. Müller *et al.* (2016) identified norovirus GI-2 from 28 out of 31 patient's faecal samples who had been implicated in an outbreak at a gala dinner. Sequence information on the polymerase and capsid region was obtained for 17 out of the 28 faecal specimens. However, from 20 lettuce heads tested only the capsid region of 1 lettuce sample was

obtained, which matched the genotype of the infected individuals. Le Guyader *et al.* (2004) has also reported no success in sequencing norovirus identified by conventional RT-PCR in raspberries implicated in an outbreak in Sweden, and even tried to attempt to clone the PCR fragment several times to no success. Due to the low viral loads and the complexity of food testing, characterisation by genotyping is difficult. With these low success rates of characterising norovirus from foods this is contributing to the lack of visibility of norovirus and other virus circulating strains in the food chain.

1.10. Aims and objectives

The objective of this PhD thesis was to develop a rapid, semi-automated and robust method that is able to detect norovirus from a range of food matrices, fomites and food handlers. The specific aims were:

- Develop, optimise and validate broadly reactive virus capture and concentration methods to recover norovirus from a range of complex ready-to-eat food matrices
- To develop a protocol to track and quantify norovirus transfer from food handlers hands to food during preparation

2. Materials and Methods

2.1. Terminology and process controls

Terms and definitions used throughout this study are summarised in Table 3.

Table 3 Definitions and terminology used throughout this thesis

Name	Definition
Ambient temperature	Incubation of samples at 22^{o}C ± 0.5^{o}C
Food surface wash	PBS wash used to elute virus from the food surface. In the final method the volume is 50ml at pH 3.5.
Ready to Eat	Food intended by the producer or manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level the micro-organisms of concern Regulation (EC) NO. 2073/2005).
Limit of detection	The lowest concentration of target norovirus in a sample that can be reliably measured by an assay. Norovirus detection was conducted either qualitatively or quantitatively determined by either a band of the correct size on a gel by gel electrophoresis or a Cq value of less than 40 by real time RT-PCR.
Inoculum	200µl of a norovirus positive faecal specimen prepared in PBS.
Region C typing assay	A region of the norovirus genome encoding the major capsid protein (ORF2) between nucleotides 5331-5653 (GenBank accession number M87661) for norovirus GI and between nucleotides 5058-5401 (GenBank accession number X86557.1) for norovirus GII.
Internal control	Tissue culture grown mengovirus.
Negative control	200µL of sterile PBS target pathogen-free, which is run through all stages of the analytical process.
Capture control	50ml of PBS spiked with inoculum and 100µl PGM coated beads from the stock solution.
Negative bead control	100µl of PGM coated magnetic bead from the stock solution added to lysis buffer.
Extraction control	200µl of mengovirus.
Negative PCR control	5µl of nuclease free water.
Internal positive PCR control	5µl of mengovirus cDNA generated using random primers.
Double stranded DNA control	Purified plasmids carrying the norovirus target sequence per genotype diluted in 1x Tris-EDTA (pH 8.0) and stored at -20^{o}C. Ten-fold dilutions from 1 x 10^{5} cDNA copies per µl to 10 cDNA copies per µl were prepared and used to generate the standard curve.

2.1.1. Faecal sample collection and preparation

Norovirus positive faecal specimens were selected from the specimen archive held at the Enteric Virus Unit, Public Health England (PHE) Colindale, London (Table 4). All specimens were prepared in a class 2 microbiological safety cabinet except during centrifugation. A 10% suspension (w/v) was prepared using 4g of faecal specimen suspended in a final total volume of 40ml of balanced salt solution (Life Technologies, Paisley, UK). The suspension was mixed by vortexing for 15 seconds and clarified by centrifugation at 1,500g in a benchtop centrifuge for 5 minutes (Fisher Scientific, Loughborough, UK). All specimens were then stored at 4^{o}C.

Table 4 A table of the faecal specimens used, the date the sample was received the, the origin and the Cq value.

Strain Name	Sample received in laboratory	Origin	10% emulsion Cq value[1]
GI-3	20.03.2013	Human faeces	16.31
GI-6	29.04.2015	Human faeces	20.90
GI-7	11.04.2013	Human faeces	20.79
GII-4	30.05.2005	Human faeces	18.01
GII-4	09.06.2015	Human faeces	29.65
GII-5	28.05.2013	Human faeces	15.51
GII-6	25.02.2015	Human faeces	18.30

[1] Cq value was obtained from the time of preparation for use in this work

2.2. Food sample collection and preparation

2.2.1. Food sample collection and preparation

The RTE foods were purchased from a local supermarket and a takeaway. A 25g portion of food was weighed on a top load balance Monoblock PB500-S1 (Mettler Toledo, Leicester, UK) into a Separator 400 Blender Bag (Grade Ltd., Leicester, UK) (PHE, 2016). The foods were selected based on the classification system in Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market (HPA, 2009) as outlined in Table 5, and the surface of these foods were contaminated with either an inoculum or a negative control.

Table 5 A List of ready-to-eat foods weighed in 25g portions (HPA, 2009) which were artificially contaminated with norovirus

Food Category		Food tested
1	Ambient stable canned, bottled, cartoned and pouched foods immediately after removal	Tuna
2	Foods cooked immediately prior to sale or consumption	Takeaway chicken and chips
3	Cooked foods chilled but with minimum handling prior to sale or consumption	Sausage rolls
4	Bakery and confectionery products without dairy cream, powdered foods	Sponge cake
5	Cooked foods chilled but with some handling prior to sale or consumption	Cooked meats
6	Non-fermented dairy products and dairy desserts, mayonnaise and mayonnaise based dressings, cooked sauces	Cream cake
7	Food mixed with dressings, dips, pastes	Salads with dressing
8	Extended shelf life food products requiring refrigeration	Salmon
9	Raw ready-to-eat meat and fish, cold smoked fish	Sushi
10	Preserved food products pickled, marinated or salted	Olives
11	Dried foods	Thyme dried
12	Fresh fruit and vegetables, products containing raw vegetables	Strawberries, Raspberries, Lettuce
13	Fermented, cured and dried meats, fermented vegetables, ripened cheeses	Cured meat

2.2.2. Food handler glove sample preparation

Two norovirus RNA positive faecal specimens, one representing each genogroup (GI-3 and GII-4) were selected and prepared as 10% suspensions as previously described in section 2.1.1. The selection criteria of the norovirus positive faecal specimens were that they contained a Cq value of less than 40 upon real time RT-PCR analysis. Food handlers were protected with a Howie laboratory coat (VWR, Leighton Buzzard, UK), a set of SHIELDskin™ Category III PPE nitrile glove (PPE Directive 89/686/EEC) (Bennekom, Netherlands) and a set of vinyl food safe gloves (PAL, Leicestershire, UK). The vinyl food safety gloves were placed on top of the nitrile gloves and were removed after use using a sterile tongue depressor (S. Murray, Surrey, UK) to prevent the glove from inverting during removal. They were placed

into a Separator 400 Blender Bag (Grade Ltd) i.e. one bag per glove, in preparation for testing, by surface washing in 50ml of PBS (pH 3.5), and the 50ml supernatant was pipetted into a 50ml tube in preparation of virus capture by PGM.

2.2.3. Environmental swabbing of food preparation surfaces

Environmental surfaces used in the food preparation areas included, two stainless steel trays approximately a 50cm x 30cm area and a 10cm x 5cm lettuce bowl. Two sterile viscose swabs (TSC Ltd, Lancashire, UK) pre-moistened in deionised water, were used to swab food preparation surfaces in parallel, each immersed in a 2ml tube (Sarstedt, Leicester, UK) containing 630µl of lysis buffer (QIAgen, Hilden, Germany). These 2ml tubes were mixed by vortexing for 15 seconds. The viscose swab was removed and discarded, and total nucleic acid was extracted from the remaining lysis buffer.

2.3. Virus concentration and capture

2.3.1. Virus concentration using 5 X Polyethylene glycol (PEG)/NaCl solution

PEG/NaCl solution was prepared by dissolving 500g of PEG x 8,000 molecular weight (Sigma Aldrich) and 87g of NaCl (1.5 mol/l) (Sigma Aldrich) in 450ml of distilled water, mixed until solids were dissolved, the total volume was then adjusted to 1,000ml with distilled water, and sterilised by autoclaving at 121^{o}C for 15 minutes. The PEG/NaCl solution was added to a final concentration of 100g/l PEG and 0.3 mol/l NaCl and incubated at 4^{o}C for one hour on a rocking platform at 60 oscillations per minute. The sample was then transferred to 50ml centrifuge tubes (Nalgene, Thermo Scientific) and centrifuged at 10,000xg and for 30minutes at 4^{o}C (Sorvall, Thermo Scientific). The supernatant was discarded and the pellet was centrifuged again at 10,000xg and for 30 minutes at 4^{o}C to form a compact pellet. The pellet was re-suspended in 500 µl of PBS and clarified further using a 1:1 ratio of

chloroform to butanol. The aqueous phase was transferred to a clean 2ml tube with 630µl of lysis buffer containing guanidium isothiocyanate (QIAgen).This was then transferred to a 2ml tube ready for extraction and was stored at 4^0C overnight before processing.

2.3.2. Virus capture and concentration using PGM conjugated to magnetic beads

Magnetic beads were coated at a final concentration of 7.5mg/ml of partially purified PGM containing 0.5-1.5% sialic acid and 0.2% *N*-Acetylneuramicacid (Sigma Aldrich), and covalently coupled with 10mg/ml of Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) supercarrier immune modulator (Fisher Scientific). Table 6 outlines the details of preparations of PGM coated beads up to 10ml batches. Magnetic beads were incubated with PGM at 4^oC for a total time of 45 minutes before separation on the MagJET magnetic rack (Fisher Scientific) for 15 minutes. The supernatant was discarded and the beads were washed twice in PBS to remove residual PGM. The PGM coated beads were stored at 4^oC and used for up to two weeks (based on a method by Tian *et al.,* 2010).

Table 6 Proportions of activated PGM coated MagnaBind™ beads required to obtain 1-10ml volumes taken from a stock solution at a concentration of 7.5mg/ml using PGM, EDC and by BupH MES buffer

BupH MES buffer (ml)	Porcine Gastric Mucin (PGM) (mg)	EDC (ml)
1	7.5	0.1
2	15.0	0.2
3	22.5	0.3
4	30.0	0.4
5	37.5	0.5
6	45.0	0.6
7	52.5	0.7
8	60.0	0.8
9	67.5	0.9
10	75.0	1

The 10% faecal suspensions prepared as described in section 2.2.1 were diluted ten-fold up to a dilution of $1:10^6$ to generate a range of viral loads determined by real time RTqPCR and 200µl of these ten-fold dilutions were used to artificially contaminate food surfaces. Contaminated food samples were surface washed in 50ml of PBS (pH 3.5), and passed through a Separator 400 Blender Bag (Grade Ltd). The food surface wash was collected and decanted into a sterile 50ml tube (Fisher Scientific) and the food was discarded.

Optimisation of the amount of PGM coated on beads was carried out using the 7.5mg/ml stock solution, the PGM was diluted in 1:500, 1:200 and 1:100 in 50ml volumes. Incubation of specimens with PGM coated magnetic beads were conducted at 4^oC, 37^oC or ambient temperature ($22^oC \pm 0.5^oC$). The food wash or glove wash samples were incubated on a rotating incubator for 45 minutes (Grant-Bio, Cambridgeshire, UK), before tubes containing the sample and magnetic bead mixture were transferred onto a MagJET magnetic rack (Fisher Scientific), for 15 minutes at 4^oC to allow the beads to separate. The supernatant was removed and discarded using a pipette. The remaining magnetic beads and captured norovirus were re-suspended in 630µl of lysis buffer (QIAgen) prepared as per manufacturer's instructions and as described in section 2.4.

2.4. Nucleic acid extraction

2.4.1. Automated nucleic acid extraction platforms and viral nucleic acid recovery

Four extraction platforms were set up as per manufacturer's instructions and according to the kit used (Table 7). These four platforms were selected based on prior suitability evaluation and analysis of performance in laboratories in PHE for the purpose of clinical

diagnostic, reference and surveillance work, for enteric viruses or food and water samples (personal communication D.Allen, N. Elviss, S.Carne). The evaluation performed in this thesis was conducted using a ten-fold dilution series of faecal specimens to assess platform sensitivity, cross contamination and throughput in a comparative platform evaluation. The specimen input volume (of the 10% faecal suspension or ten-fold dilution of the specimen) remained constant at 200µl, whist the total input volume (specimen plus lysis buffer) varied by platform according to the recommended lysis buffer volume, as indicated by the manufacturer. To limit the effects of methodological inconsistencies for the purpose of the evaluation, the elution volume was set at either 110µl or 100µl as this was the volume most similar across all platforms depending on the programme settings available for each automated platform (Table 7).

For food and glove washes, PGM coated magnetic beads were removed prior to nucleic acid extraction by adding the required volume of lysis buffer (Table 7), so the captured norovirus capsids were eluted off of the magnetic beads, vortexed for 10 seconds and centrifuged at 1,500xg for 5 minutes (Fisher Scientific) and so that the magnetic beads were pulled down and pipetting off the total input volume of sample in lysis buffer into either (1) a fresh 2ml tube (Sarstedt) for the QIAgen QIAsymphony™ or (2) a 96 well plate for the Roche Mag*NA* Pure 96™ ready for extraction. The smallest elution volume on programme settings was selected to allow for the processing of samples potentially containing low viral loads (Table 7).

Table 7 Nucleic acid extraction platform kit, lysis buffer preparation and protocols used in evaluation experiments for faecal, food and glove samples

	Faecal sample				Food and glove wash sample	
	Roche Mag*NA* Pure 96™	Qiagen QIAymphony™	Qiagen QIAxtractor™	Promega Maxwell 16™	Roche Mag*NA* Pure 96™	Qiagen QIAymphony™
Kit used	DNA and viral RNA Small volume kit	Virus pathogen mini kit	Virus Plasticware Kit	Total RNA Purification Small Elution Volume RNA kit	DNA and viral RNA Large volume kit	Virus pathogen mini kit
Extraction protocol[1]	Pathogen Universal 200	The complex200_off board LYSIS_V4_DSP	prelysis_100ul	Maxwell® 16 SEV Hardware Kit RNA Purification	Pathogen Universal 500	The complex200_off board LYSIS_V4_DSP
lysis buffer and reagent preparation	None	20µl of proteinase K 100µl of buffer ATL 190µl of buffer ACL buffer 120µl of carrier RNA (1µg/ml), internal control and buffer AVE	210 µl of RNA/Elution solution 1.1ml VX Digest 10ml VXL buffer	500µl of clearing agent	500µl of external lysis buffer	20µl of proteinase K 100µl of buffer ATL 190µl of buffer ACL buffer 120µl of carrier RNA (1µg/ml), internal control and buffer AVE
Input volume (+ lysis buffer if required)	200µl	630µl	300µl	600µl	500µl	630µl
Elution volume	110µl	100µl	100µl	100µl	100µl	60µl

[1]Extraction protocols are specific names given by manufacturers

2.4.2. Growth of Mengovirus strain MC_0 for use as an extraction control and internal process control

Mengovirus strain MC_0 (ATCC VR-1597) was kindly provided by James Lowther (Cefas, Weymouth, UK). The virus was propagated in healthy HeLa cells as described in previous methods (ISO/TS 15216, 2013). The supernatant of infected HeLa cells were harvested, the cells were pelleted at 2,000g for 10 minutes and the virus supernatant was stored in 10µl single-use aliquots. The 10µl aliquots were diluted 1:100,000 for use as an extraction control and internal process control as required.

2.5. PCR detection

2.5.1. Reverse transcriptase reaction

All reverse transcription reactions were conducted to a final volume of 70µl comprised of 40µl of total nucleic acid and 30µl of reverse transcriptase mix. The RNA strands were denatured at 95^{o}C for 5 minutes before incubation at 37^{o}C for one hour with 1x PCR buffer (Invitrogen), 10mM $MgCl_2$ (Invitrogen), 2mM each dNTP (Invitrogen) and 458U of Mu-MLV Reverse Transcriptase (Invitrogen). The reaction was terminated by incubation at 95^{o}C for 2 minutes before being snap cooled on ice for 5 minutes. Excess cDNA was stored at -20^{o}C.

2.5.2. Detection of norovirus using the Kageyama two-step assay

This was performed in a 25µl total reaction volume, comprised of 1x Platinum RT-PCR UDG Supermix (Invitrogen), 0.4mM of each genogroup specific forward and reverse primer (Invitrogen), 0.1mM probe (Invitrogen), 1x ROX dye (Invitrogen) and RNase-free water (Invitrogen). All probes used in norovirus genogroup detection were labelled at the 5' end with FAM and 3' end with TAMRA. Thermal cycling conditions were 95°C for 10 minutes

followed by 40 cycles at 95°C for 15 seconds and 56°C for 1 minute. Oligonucleotide primers and probe were used as defined in the published paper by (Kageyama *et al.,* 2003) (Table 8).

Table 8 Primers and probes targeting ORF 1-2 junction for genogroup detection by Kageyama *et al.,* (2003) assay

Norovirus strain	Reagent name	Sequence (5'-3')	Amplification size (bp)	Original reference
GI	Cog1F (forward primer)	CGYTGGATGCGNTTYCATGA	105	(Kageyama *et al.,* 2003)
	Cog1R (reverse primer)	CTTAGACGCCATCATCATTYAC		
	Ring 1(a)TP (probe)	FAM-AGATYGCGRTCYCCTGTCCA-TAMRA		
GII	Cog2F (forward primer)	CARGARBCNATGTTYAGRTGGATGAG	98	(Kageyama *et al.,* 2003)
	Cog2R (reverse primer)	TCGACGCCATCTTCATTCACA		
	Ring 2TP (probe)	FAM-TGGGAGGGCGATCGCAATCT-TAMRA		

2.5.3. Detection of norovirus using the Le Guyader one-step assay

Norovirus RNA was detected in total nucleic acid extracts obtained from specimens extracted using the QIAgen QIAsymphony™ using a real time RT-PCR published by Le Guyader *et al.,* (2009). This assay had a final reaction volume of 25µl comprised of 5x RNA Ultrasense mix (Invitrogen), 0.5mM genotype specific forward primer (Invitrogen), 0.9mM genotype specific reverse primer (Invitrogen), 0.25mM Probe (Invitrogen), 1x ROX dye (Invitrogen) and RNase-free water (Invitrogen). All probes used specifically for norovirus detection were labelled at the 5' end with FAM and 3'end with TAMRA. Thermal cycling conditions were 55^{o}C for 1 hour and 95^{o}C for 5 minutes, followed by 95^{o}C for 15 seconds, 60^{o}C for 1 minute 65^{o}C for 1 minute for 45 cycles using oligonucleotide primers and probes were used as defined in the published paper by Le Guyader *et al.,* (2009) (Table 9).

Table 9 Primers and probes targeting ORF 1-2 junction for genogroup detection by Le Guyader *et al.,* (2009)

Norovirus strain	Reagent name	Sequence (5'-3')	Amplicon size (bp)	Original Reference
GI	QNIF4 (forward primer)	CGCTGGATGCGNTTCCAT	86	(da Silva *et al.,* 2007)
	NV1LCR (reverse primer)	CTTTAGACGCCATCATCATTTAC		(Svraka *et al.,* 2007)
	NVGG1p (probe)	FAM-TGGACAGGAGAYCGCRATCT-TAMRA		
GII	QNIF2 (forward primer)	ATGTTCAGRTGGATGAGRTTCTCWGA	89	(Loisy *et al.,* 2005)
	Cog2R (reverse primer)	TCGACGCCATCTTCATTCACA		(Kageyama *et al.,* 2003)
	QNIFs (probe)	FAM-AGCACGTGGGAGGGCGATCG-TAMRA		(Loisy *et al.,* 2005)

2.5.4. Bioinformatic analysis

Analysis of the primers and probes used in real time RTqPCR assays was undertaken *in silico* by mapping all genogroup I primer and probe combinations against the whole genome of reference strain GI-I (GenBank accession number M87661) and all genogroup II primer and probe combinations against a whole genome of reference strain GII-4 (GenBank accession number X86557.1) in BioEdit. Primer sets used in the Le Guyader *et al.* (2009) assay are described throughout this thesis as the Le Guyader primers, however the original reference can be found in Table 9. The RT-PCR reactions were performed on a 7500 Fast TaqMan instrument in the fast mode (Applied Biosystems).

2.6. Quality controls

2.6.1. Detection of Mengovirus strain MC_0 extraction control and internal process control

Total nucleic acid from a 200µl aliquot of a 1:100,000 dilution of Mengovirus MC_0 was extracted on each extraction, as an external extraction control. Additionally, Mengovirus was used as an internal process control in which 16µl of the 1:100,000 dilution was spiked into each specimen prior to extraction. Mengovirus cDNA was detected by real time RTPCR. Monoplex PCR reactions were performed in a 25µl total reaction volume, comprised of 1x platinum real-time PCR UDG supermix (Invitrogen), 0.5µM forward and 0.9µM reverse primer (Invitrogen), 0.1µM probe (Invitrogen), 1x ROX dye (Invitrogen) and RNase-free water (Invitrogen). The probe used for detection of mengovirus was labelled at the 5' end with FAM and 3'end with MGBNFQ (minor groove binder/ non-fluorescent quencher). The thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 56°C for 1 minute using oligonucleotide primers and probes from a published paper (Pinto *et al.,* 2009). The sequence of the primers and probe used were as stated in Table 10. The Cq value was recorded and the selection criteria of ±3 standard deviations from the average Cq values in a validated dataset were set as an extraction quality control check (Appendix A).

Table 10 Primers and probe targeting 100bp region of Mengovirus the MC_0 strain

	Reagent name	Sequence (5'-3')	Original Reference
Mengovirus MC_0	Mengo 110 (forward)	GCGGGTCCTGCCGAAAGT	(Pinto *et al.,* 2009)
	Mengo 209 (reverse)	GAAGTAACATATAGACAGACGCACAC	
	Mengo 147 (probe)	FAM-ATCACATTACTGGCCGAAGC-MGBNFQ	

2.6.2. Norovirus positive real-time PCR controls

Norovirus genogroup I genotype 3 where a GI virus was used as a contaminating strain and genogroup II genotype 4 cDNA controls were used where a GII norovirus was used as a contaminating strain. A selection criterion of ±3 standard deviations from the average Cq values in a validated dataset was set as a quality check for the Cq value detected in the cDNA controls in every PCR experiment (Appendix B).

2.6.3. Real time PCR and quantification standards

Cq values were recorded as a proxy for viral load, for experiments up to section 3.3.6. A difference greater than 3.3Ct or 1 log between two specimens was considered significant. For all other experiments quantification was determined using a standard curve. The standard curve was generated as defined in Table 3. Data generated for standard curves were analysed to detect issues which may affect the quantitiative value assigned to a positive specimen. Any points of the standard that fell outside of the line of best fit were discarded. For both norovirus GI (Appendix C) and norovirus GII (Appendix D) the Cq values of the standards were recorded for a high viral load ($1x10^4$ cDNA copies/µl) and low viral load ($1x10^1$ cDNA copies/µl) in every PCR experiment for quality purposes. To avoid damage to the standard curve material by freeze thawing, single use aliquots of each dilution were used on every RT-qPCR assay run. Each dilution used to generate the standard curve was added to the RT-qPCR plate in duplicate. The standard was then used to generate the number of cDNA copies per total nucleic acid extract.

To normalise the data between the one-step Le Guyader assay and the two-step Kageyama assay, the number of cDNA copies per µl of faecal inoculum was calculated using the specific equations:

The Le Guyader assay calculations:

cDNA copies per total nucleic acid extract (or per inoculum) =

Number of cDNA copies per µl of reaction x 60µl of total nucleic acid extract

Conversion of cDNA copies per 0.02g gram of faeces into cDNA copies per gram of faeces=

cDNA copies per 0.02g gram of faeces x (1/dilution factor)

The Kageyama assay calculation:

cDNA copies per total nucleic acid extract (or per inoculum) =

No. of cDNA copies per µl of reaction x 40µl x 1.5µl

Conversion of cDNA copies per 0.02g gram of faeces into cDNA copies per gram of faeces=

cDNA copies per 0.02g gram of faeces x (1/dilution factor)

2.7. Statistical analysis

All real time RTqPCR data was analysed in the ABI 7500 Taqman SDS software (Applied Biosystems). The data was exported to Microsoft Excel version 2010 for further analysis. A one way ANOVA test was conducted to address the aim to identify whether the average number of norovirus cDNA copies detected from faecal specimens was statistically significant between the Kageyama *et al.,* (2003) PCR assay and the Le Guyader *et al.,* (2009) PCR assay. An F-test was conducted to determine whether variance between contamination on the left non dominant and the right dominant hand was equal, followed by a two tailed T-test to identify if the mean number of cDNA copies recovered on the left non dominant and the right dominant hand was statistically significant for either GI or GII food handler simulations. Descriptive statistics were calculated on two or more replicates

specimens, these included the calculation of 95% confidence intervals, standard deviations and averages all calculated by Microsoft Excel version 2010.

2.8. Virus Characterisation in food handling experiments

2.8.1. Norovirus typing Algorithm

For all specimens in food handling experiments the characterisation algorithm was followed as described in Figure 5.

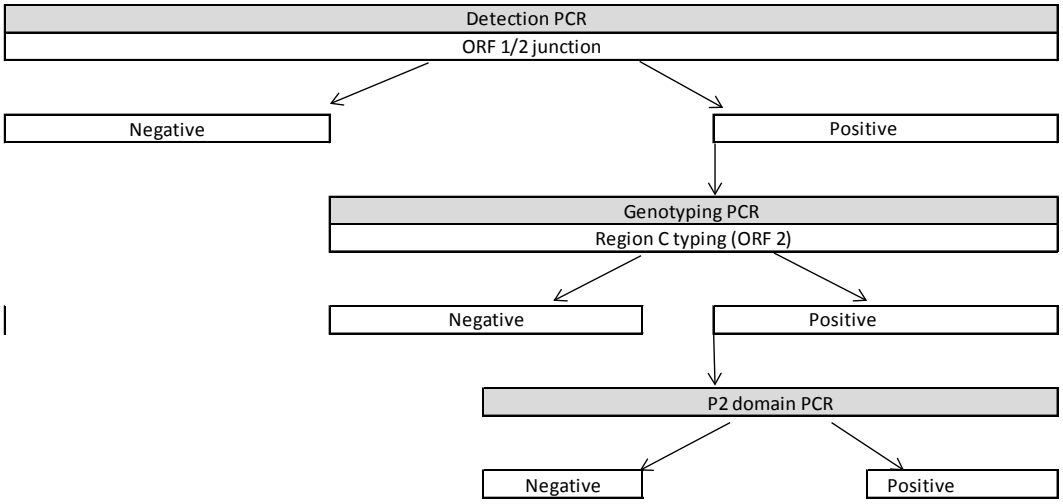


Figure 5 A norovirus characterisation algorithm

Noroviruses genotypes were characterised by amplification and sequencing of region C (Sanger *et al.,* 1977), and GII.4 viruses were further characterised by amplification and sequencing of the hypervariable P2 domain (Xerry *et al.,* 2008; Allen *et al.,* 2008). Amplification of 340-370bp of region C; the capsid gene within the S domain of the norovirus genome, was conducted using a semi-nested PCR. The thermal cycling conditions were a denaturation step of 94°C for 1 minute, followed by 40 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute and one cycle at 72°C for 5 minutes in the first round PCR reaction. Second round PCR reactions were conducted by a denaturation step of 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 1 minute,

72°C for 1 minute, and a single cycle at 72°C for 5 minutes. These PCR total reaction volumes of 50µl comprised of 20µM of forward and reverse primer (Invitrogen), 1x PCR buffer (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 2.3mM dNTPs, 2U of *Taq* Polymerase (Invitrogen) and molecular grade water (Severn Biotech, UK) using oligonucleotide primers from published papers (Kojima *et al.,* 2002, Gallimore *et al.,* 2005). The sequence of the primers and probe used were as stated in Table 11.

Table 11 Primer sequences used in amplification of: first or second round PCR products in the region C typing assay

Round	Primer name	Sequence (5'-3')	Amplicon size (bp)	Original Reference
First	GI FFN	GGAGATCGAATCTCCTGCCCC	364	(Kojima *et al.,* 2002) (Gallimore *et al.,* 2005)
	GI SKR	CCACCCCACCATTRTACA		(Kageyama *et al.,* 2003)
Second semi-nested	GI FFN2	ATCTCCTGCCCGAWTWYGTAA	343	(Gallimore *et al.*, 2005, Kojima *et al.*, 2002)
First	GII FBN	TGGGAGGGCGATCGCAATCT	364	(Gallimore *et al.,* 2005)
	GII SKR	CCRCCNGCATRHCCRTTRTACAT		(Kageyama *et al.,* 2003)
Second semi-nested	GII FBN2	GCGATCGCAATCTGGCTCCC	343	(Kojima *et al.*, 2002)

For further characterisation by amplification of the P2 domain, amplification of 790bp was conducted in the first round PCR and 512bp in the second round PCR of the P2 domain. This was set up in a total reaction volume of 50µl which comprised of 1x PCR Reaction Buffer (Invitrogen), 1mM each dNTP (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 20pmol of forward and reverse primer (Invitrogen), and 2U of *Taq* Polymerase (Invitrogen) and molecular grade water (Severn Biotech, UK). The amplicons were generated by a denaturation step of 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C

for 1 minute for 40 cycles, followed by an extension step of 72^{o}C for 5 minutes in the first round PCR reaction. Second round PCR reactions were conducted by a denaturation step of 94^{o}C for 2 minutes, followed by 94^{o}C for 30 seconds, 53^{o}C for 1 minute, 72^{o}C for 1 minute for 40 cycles and an extension step of 72^{o}C for 5 minutes using nucleotide primers from published papers (Xerry *et al.,* 2008). Amplification of the P2 domain for other genotypes used the same 50µl reaction mix; however, genotype specific oligonucleotide primers and annealing temperatures were adjusted as outlined in Table 12.

Table 12 Primers and annealing temperatures for generation of both first and second round PCR products of the P2 domain domain GII-4 specific PCR assay, for specific norovirus GI and GII genotypes used in validation experiments of the intra-genotyping assay

Norovirus genotype	Round	Primer names	Primers (5'-3')	Amplicon size (bp)	Annealing temp °C	References
GI-3	First round Second round semi nested	P2 GI-3 F P2 GI-3 R P2 GI-3 FN	TCWAAYTCAAGRGTCCCTTCT GCTTCMCCTCTAGTGGGGGCCT TTCWYTMATYAAWKCWATGAT	682 662	50 40	(Xerry *et al.,* 2010, Xerry *et al.,* 2008)
GI-6	First round	P2 GI-6 F P2 GI-6 R	TCAAATTCTCGTGTCCCTGTG GTTCATTRCAGAAGTGGGTAAT	646	45	(Xerry *et al.,* 2010)
GI-7	First round	P2 GI-7 F P2 GI-7 R	GCTAACTCCAGAGTGCCCGCA GCGGCTTCACCTCGGATTGGTG	674	50	(Xerry *et al.,* 2010)
GII-4	First round	GII 4P2F GII 4P2R	GANGATGTCTTCACAGTCTCTT CATTCCTGGGGGAGTAGACA	790	53	(Xerry *et al.,* 2008)
	Second round nested	N-P2F N-P2R2	TGGCARTGYACGACTGATGG CTRAAGAAAAGAAGYTGCTCA	512	50	(Allen *et al.,* 2008)
GII-5	First round	P2 GII-5 F P2 GII-5 R	GACGCAGTTTTCACCGTCTCAT CACTCCTGAGGCACCAGACA	794	48	(Xerry *et al.,* 2008)
GII-6	First round Second round semi nested	P2 GII-6 F P2 GII-6 R P2 GII-6 FN	GAGGACGTGTTCACTGTTTCTT CATTCCTGGGGTATGAGACA CACCAACTGTTGAATCAAAAA	827 753	45 45	(Xerry *et al.,* 2008)

All PCR products were resolved on a 1% (w/v) agarose gel by electrophoresis. Gels were made using Agarose MP (Roche Diagnostics Ltd) and 1x Tris-Borate EDTA (TBE) (Invitrogen). Electrophoresis was performed at 110V/cm^2 on a 13.2cm x 14.2cm gel, for 100 minutes. Products were visualised after immersion of the gel in an ethidium bromide tank containing 5mg/l ethidium bromide (Sigma Aldrich) in 1x TBE buffer (Invitrogen) for 15 minutes. The gel was washed three times with water and visualised under a UV light using the GelDoc system (BioRad Laboratories Ltd, Hertfordshire). The amplicons were measured against a 100bp ladder (Invitrogen) run in parallel on the agarose gel. Amplicons of the correct size were purified from solution using the AMPure Agencourt® system. Amplicons were purified using 65µl of Agencourt® Ampure®magnetic beads (Beckman Coulter, UK) added to 45µl of the PCR product in solution in a 96 well plate. Agencourt®® Ampure®magnetic beads and PCR products were mixed ten times by pipette until the colour of the mixture appeared homogenous. The homogenous sample was then incubated at ambient temperature for three minutes. The 96 well plate was placed on the Agencourt® SPRIplate 96R (Beckman Coulter) and allowed to incubate for five-ten minutes. The colourless solution was aspirated and discarded and 200µl of 70% ethanol was added to each reaction well and incubated at ambient temperature for 30 seconds. The ethanol was aspirated, discarded and this was repeated two more times. Once the ethanol was removed the 96 well reaction plate was air-dried for 15 minutes at ambient temperature before 40µl of RNase free water was added to elute the DNA. Excess purified PCR product was stored at -20^{o}C.

DNA was set up to be sequenced in an ABI MicroAmp optical 96 well plates (Applied Biosystems) by the addition of 2pmols/µl of primer and 30ng of purified DNA template to a total reaction volume of 6µl for each sequencing reaction (Sanger *et al.,* 1977). All

sequencing was performed by PHE in-house genomics and sequencing service using BigDye Chemistry version 3.1 (Applied Biosystems) and analysed by the Genetic Analyser 3730XL (Applied Biosystems). Nucleotide sequence contigs were generated from trace sequence data using the Assembler tool in seqMan Pro (DNASTAR version 12.2), ClustalW multiple alignment and phylogenetic analysis was also performed using algorithms in MegAlign (DNASTAR version 12.2). For region C typing, contig sequences were trimmed to position 5331 at the 5' and position 5653 at the 3' end, and the trimmed sequences were aligned by ClustalW multiple alignment in MegAlign. The P2 region defined as ranging from amino acid 279 to 405 (Prasad *et al.,* 1999), was then used to confirm sequence homology of the P2 domain. Contig sequences were trimmed to these amino acid positions, and the trimmed sequences were aligned by ClustalW multiple alignment in MegAlign.

2.9. Simulation experiments

2.9.1. Food handling experimental design

A traceable ultra violet (UV) product (KlerReveal Caerphilly, UK), applied as a cream to the gloved hand of food handler volunteers was used in preliminary experiments as a proxy for norovirus contamination. The transfer of UV cream was visualised on the gloved hands of the food handlers, the food and in the food preparation environment. The transfer of the UV cream was visualised under the 4Watt UV torch (KlerReveal) at each stage of sandwich making.

All sandwich preparations were conducted in a class 1 microbiological safety cabinet, on a sterile 20cm x 10cm preparation tray and stock tray and a lettuce bowl 7cm x 5cm set out as shown in Figure 6.

Food was weighed as described in section 2.2.1, and consisted of one slice of cheese, two slices of bread and a lettuce leaf. Food handlers were randomly selected from a pool of 20 volunteers (all laboratory staff at PHE). Only right handed volunteers were selected for consistency in food handling tasks.

Figure 6 Food handling simulation preparation tray in a class 1 microbiological safety cabinet.

Volunteers were not aware whether their left hand was being contaminated with inoculum, or a 200µl of water as a mock. This was anonymised to encourage consistent food handling behaviour. The use of the 200µl of water as a mock was conducted as a negative control in-between food handling simulations. The process of preparing a sandwich was used and segregated across three food handler volunteers as described in Figure 7. One simulation experiment was completed once all three volunteers had conducted their allotted tasks.

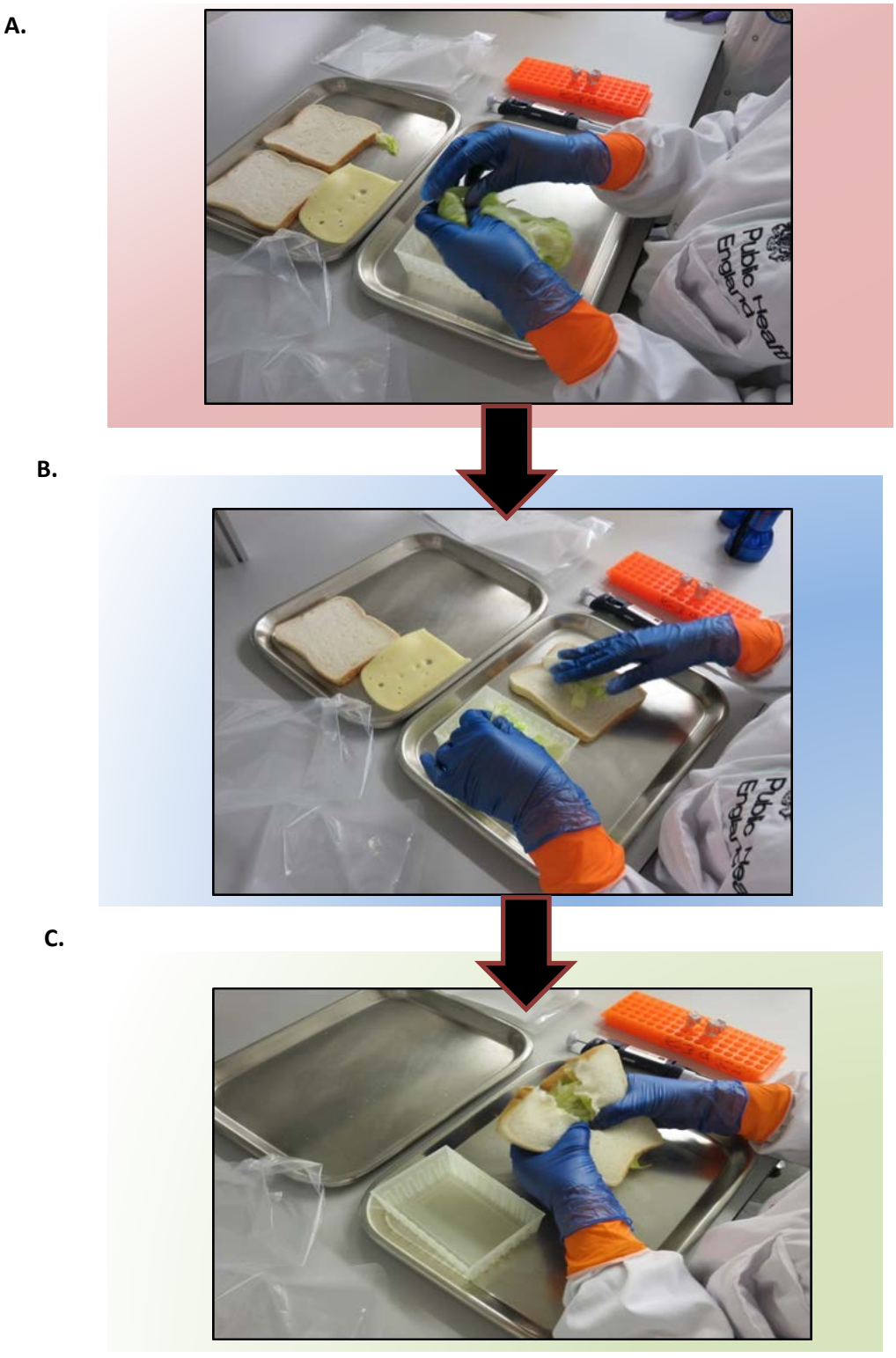


Figure 7 A. Food Handler 1 conducting task 1: inoculated 40µl of inoculum pipetted onto each fingertip of non dominant (left hand) and transfers to the right hand by pressing fingertips together for 20 seconds. B. Food Handler 2 conducting task 2: assembled the sandwich using one slice of cheese, two slices of bread and all the 2cm by 2cm lettuce pieces diced by Food Handler 1. C. Food Handler 3 conducting task 3: to segregate the sandwich into two by hand and to place each half into a different Stomacher® bag

3. Validation of a method of detecting viruses from food

3.1. Background

A rapid, robust and, where possible, automated method for detecting viral pathogens in complex food matrices is required, to enhance public health and recommendations during foodborne outbreak investigations. Viral contamination of foods is typically associated with low viral loads and the optimisation of a procedure to enhance recovery of viral genomes from food is required. Processing of the food at the beginning of the detection process is complicated by the wide variation of ingredients, and the surface textures of different food types. Therefore, a sample preparation method which is widely applicable and will increase sensitivity of detection from a range of food matrices is required. In some foods such as fresh produce, the perishability can alter the integrity of these matrices, adding to the complexity of food testing. The variation in pH determined by different foods may also cause interference with the chemistry used in virus capture, concentration and nucleic acid extraction systems. As norovirus is a non-enveloped virus it is fairly resistant to extreme environmental conditions such as extreme pH ranges. There are published methods that can be used to normalise the pH of the food to aid flocculation of virus particles from food surfaces and improve virus capture and concentration efficiency. These include the use of tris-glycine, tris-sodium chloride and beef extract buffers, to increase the pH of the food surface wash to 9.5 (Butot, 2007; Baert *et al.,* 2008; Park *et al.,* 2010). Hydrochloric acid has then been used to neutralise the food surface wash to pH 7.0 during the virus capture and concentration stages (Boxman *et al.,* 2017). There are also various methods available to capture and concentrate viruses. Specifically, PGM conjugated to magnetic beads has been used to capture norovirus; this method exploits the affinity and relationship between the norovirus P2 domain and glycans, HBGAs present in human cell surfaces

and bodily fluids of secretor individuals. The first studies to determine these interactions were through crystallography of norovirus GII-4 VA387 (Cao *et al.,* 2007). Following this study, the interactions between different HBGA phenotypes and specific norovirus strains were investigated in *in vitro* experiments using VLPs as a surrogate for norovirus virions and saliva as a source of HBGAs (Harrington *et al*., 2004, Hutson *et al.*, 2002, Huang *et al*., 2005, Donaldson *et al.*, 2008). A limitation of using saliva is that to represent many different HBGA phenotypes, a large panel of saliva samples are required. Saliva contains many carbohydrate compounds of unknown structure and different HBGAs, depending on the individual from which it is sourced. The role of these unknown compounds in the binding of norovirus remains unknown. Alternative products are synthetic oligosaccharides, which can be purified to contain a single synthetic HBGA. This is advantageous when used in studies to establish specific binding affinities, as the structure of the carbohydrate compound is defined (Huang *et al.,* 2005, Donaldson *et al.,* 2008). However, inconsistencies in assay sensitivity between studies were reported (Donaldson *et al*., 2008, Harrington *et al*., 2004, Cannon and Vinje, 2008, Lindesmith *et al.,* 2008). PGM is a readily available commercial product that contains HBGAs, specifically A, H and Le^y antigens. *In vitro* studies have been conducted using PGM and identified that the binding relationship between the HBGAs contained in this product, and different genotypes of norovirus using VLPs (Tian *et al*., 2007b). The presence of these antigens has also been implicated in human and porcine norovirus infection (Tian *et al*., 2007b, Shirato, 2011). The purpose of virus capture and concentration not only addresses the issue of recovering low viral loads from large sample volumes, it is required to concentrate sample volumes down to a level suitable for nucleic acid extraction.

There are many types of nucleic acid extraction systems, however most modern extraction chemistries are based on the silicia guanidinium isothiocyanate method developed by Boom *et al.* (1990). Choosing a nucleic acid extraction method requires the consideration of many factors, including the analyte type, the structure of the target (RNA or DNA genomes), sample throughput, cost, and extraction performance. In a study by Knepp *et al.*, (2003), an evaluation of the manual QIAmp Kit™ was compared to the automated Biorobot and Roche Mag*NA* Pure 96™, for the detection of enterovirus RNA. Enterovirus RNA was detected in 10/15, 11/15 and 11/15 clinical samples respectively, demonstrating comparability in extraction platform performance. Although the sensitivity of automated and manual extraction methods may be comparable, automated extraction methods provide the additional benefit of processing specimens consistently; reducing human error compared to processing by manual methods. Depending on the extraction system, automated methods of RNA extraction allow for approximately two to three times the number of specimens to be processed in a given time period compared to manual methods (Witlox *et al.,* 2008). With an existing throughput capability of at least 30,000 specimens being processed in each of the Official Control Laboratories for food in England each year, automation was incorporated into the validation process where possible. For these reasons manual methods of extraction were excluded from the evaluation.

In a comparison study of five different automated extraction platforms, norovirus RNA was detected in 36/39 faecal samples extracted on the Roche Mag*NA* Pure 96™ and QIAgen QIAsymphony™ (Verheyen *et al.*, 2012). Witlox *et al.* (2008) also reported no statistical significance in the extraction of norovirus RNA from faecal samples by the automated platforms Roche Mag*NA* Pure 96™ and Corbett X-tractor gene™ extraction platforms. But extrapolation of data obtained with faecal samples to different variability

of efficacy of nucleic acid extraction methods is dependent not only on the organism to be detected but also on the matrix in which it is contained.

Finally, norovirus RNA detection will depend on the amplification efficacy. For detection of norovirus and hepatitis A in bivalve mollusks, soft fruits and leafy green vegetables, one specific primer and probe set in a specific assay format has been validated thoroughly (Le Guyader *et al.,* 2009). However, other assays have been validated for use in clinical laboratories for the detection of norovirus from faecal samples (Kageyama *et al.,* 2003).

This chapter describes the optimisation of a capture and concentration method for noroviruses from food matrices, followed by an automated nucleic acid extraction method and RT-PCR that can be used as a reproducible single protocol that can be applied to a variety of foods in a high throughput food and environmental microbiology laboratory setting.

3.2. Food sample preparation optimisation

3.2.1. Sample preparation: homogenisation compared to surface washing for food samples

Virus contamination of foods is unevenly distributed; two different food sample preparation methods, surface washing and homogenisation were compared for their efficacy in recovering norovirus from food contaminated with a norovirus positive faecal sample. Three different RTE food categories were selected from The Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market (HPA 2009). These three food categories were selected to provide different food compositions for artificial contamination.

All foods were weighed in 25g portions and spiked with norovirus in a 200µl volume on as a single spot wet inoculum on the food surface, and incubated for 30 seconds in a laminar flow hood. Spiked foods were subjected to surface washing or homogenisation in total volumes of 50ml of PBS (pH 7). All experiments were conducted in duplicate and the real time RT-PCR Cq values compared (Table 13). For all food items tested with the exception of lettuce, surface washing outperformed homogenisation, as demonstrated by the significantly lower Cq values obtained with this method, which corresponded with 1 to two log increase in norovirus RNA recovery. This led to the decision that all foods would be surface washed in future validation experiments.

Table 13 A table presenting the Cq values of norovirus GII recovery from five foods selected from three RTE food categories (HPA 2009) to evaluate the surface wash and homogenisation methods of food processing *=significant by one to two logs difference or **=significant by greater than two logs difference

Ready-to-Eat Food Category HPA (2009)[1]		Average Cq of food surface wash conducted in duplicate on samples (Cq)	SD	Average Cq of food homogenised conducted in duplicate on samples (Cq)	SD
5	Cooked meat	22.0*	0.2	26.8	7.3
12	Lettuce	24.0	3.1	26.0	8.1
12	Raspberries	26.3*	3.5	32.5	9.1
13	Cured meat	22.5**	2.7	29.5	6.3
13	Cured meat	22.0**	2.4	31.8	8.2

[1] Refer to table 5 for full description of RTE food categories

3.3. Optimisation of the virus capture

The ISO/TS 15216 (2013) method uses PEG/NaCl precipitation and high-speed centrifugation for virus concentration. Another broadly reactive concentration method previously described uses PGM conjugated to magnetic beads (Tian *et al.,* 2010). To determine the sensitivity of these two approaches and the ability to concentrate

norovirus from a large volume, three tenfold dilutions of a norovirus GII-5 positive faecal specimen were made and single aliquots of each dilution was spiked into six 50ml samples of PBS (pH7). PBS was selected as a sample type presenting no or little inhibition, so that the sensitivity of the two methods to capture norovirus from a large volume could be compared. The concentration methods were followed as described in Figure 8 and both methods were compared based on processing time and throughput capability. Half of the 50ml specimens were concentrated using PGM and the other half using precipitation method described in ISO/TS 15216 (2013) (Figure 8). The ISO/TS 15216 (2013) method total processing time was 3 hour 40 minutes compared to 1 hour 20 minutes for the PGM capture method. The throughput capability of the ISO/TS 15216 (2013) method was limited by the volume (72.5 ml sample + PEG/NaCl) and capacity of the rotor (6 x 50ml maximum) so only 3 specimens could be processed at a time. The PGM capture method allowed twelve 50ml samples to be processed every 15 minutes on the MagJet Magnetic rack (Figure 8). This was four times the number of 50ml food specimens compared to ISO/TS 15216 (2013). The sensitivity between the two methods was not significantly different as demonstrated (Table 14). However this data was obtained from a small sample set, conducted with dilutions of norovirus GII-5 with a view to conduct replicates and explore other norovirus genotypes in further experiments. However, due to health and safety concerns involving the use of chloroform in ISO/TS 15216 (2013) it was not possible to continue experiments using this method. The PGM method was chosen for further validation work due to the time the 2 h and 20 minute savings this approach afforded, and the absence of chloroform use with this method.

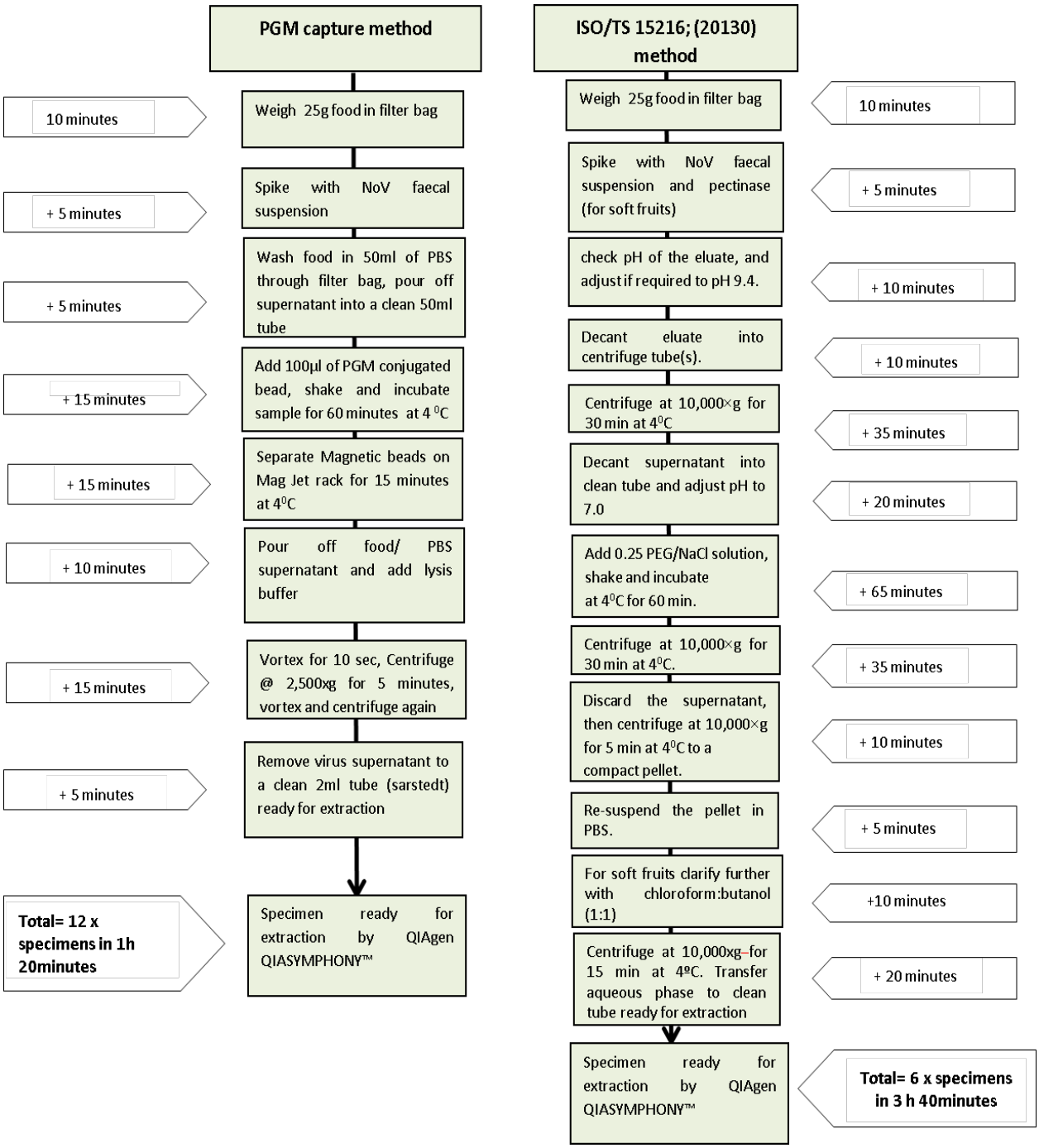


Figure 8 Step by step work flow of the time taken for the elution of virus from the surface of foods, and the capture and concentration of norovirus using two different concentration methods; PGM conjugated to magnetic beads (Tian *et al.,* 2010) or ISO/TS 15216: (2013), in preparation for nucleic acid extraction.

Table 14 Cq values of norovirus GII-5 inoculum diluted in ten-fold dilutions, spiked in 50ml of PBS concentrated using PGM method or ISO/TS 15216 (2013) method from one sample

norovirus GII dilution series	Tian *et al.* (2010)	ISO/TS 15216 (2013)
10% suspension	22.5	21.4
1:10	26.4	25.4
1:100	29.7	29.7

3.3.1. Capture: experiments to determine specific and non-specific capture using PGM

In order to establish the specificity of the PGM capture method, non-activated non-coated magnetic beads (as provided by the manufacturer), EDC activated non-coated beads; BSA activated coated beads, and PGM activated coated beads were tested with a tenfold serial dilution of a norovirus GII sample. This was diluted again 1:250 in a total volume of 50ml. A test bead preparation was added to a 50ml sample, and processed as previously described. The results demonstrated that all test beads captured norovirus in a non-specific manner, but the PGM coated beads recovered >1 log more than the rest (Table 15). Due to non-specific capture taking place, capture of an unrelated virus, mengovirus was tested at the dilution used in the internal process control (1:100,000). Mengovirus was not captured by PGM coated beads (data not shown).

To establish efficiency of the PGM capture norovirus recovery was measured from a total of 200µl (0.4%) of the 50ml sample, the beads and 200µl of the 49.8ml sample

supernatant post –bead capture. The results demonstrated norovirus RNA was detected only from the two highest concentrations in 200µl (0.4%) of the 50ml samples, no viral RNA could be detected in the supernatants post capture (Table 16). PGM capture allowed the detection of viral RNA in all dilutions, and Cq values were consistent with the dilution factor.

Table 15 Average Cq value of norovirus GII inoculum and norovirus GII captured by non-coated non-activated magnetic beads, activated PGM coated magnetic beads, BSA coated magnetic beads, and partially activated EDC coated magnetic beads. Experiments were done in triplicate ± 95% CI. nvd=no virus detected, n/a=not applicable

GII norovirus inoculum	Inoculum (average Cq)	±95% CI	non-activated non-coated beads (average Cq)	±95% CI	EDC activated non-coated beads(average Cq)	±95% CI	BSA activated coated beads (average Cq)	±95% CI	PGM activated coated beads (average Cq)	±95% CI
10% suspension	15.2	0.1	20.1	0.8	21.3	6.6	23.4	0.5	17.4	0.6
1:10	16.4	0.3	26.3	0.9	26.8	3.7	23.4	0.5	22.7	0.4
1:100	19.9	1.0	29.2	2.4	28.1	4.7	25.4	0.1	24.1	0.1
1:1,000	23.7	1.0	31.8	3.1	31.6	3.6	27.6	0.3	27.8	1.7
1:10,000	26.8	1.6	nvd[1]	n/a[2]	33.6	2.3	31.6	3.0	30.7	1.7
1:100,000	29.6	2.8	nvd	n/a	37.2	1.3	nvd	n/a	33.7	2.0

[1] nvd=no virus detected [2]n/a=not applicable

Table 16 percentage of inoculum recovered from 200µl or 0.4% of the 50ml sample tested, 49.8ml sample concentrated by PGM coated beads, and 200µl of the 49.8ml sample supernatant- post concentration by PGM coated beads(±95%CI)**=significant, greater than two logs difference.

Norovirus GII inoculum (ten-fold dilution)	200µl or 0.4% of the 50ml sample (% of spiked norovirus inoculum recovered)	±95 %CI	49.8ml sample concentrated by PGM and eluted from magnetic beads (% of spiked norovirus inoculum recovered)	±95 %CI	200µl of the 49.8ml sample supernatant- post concentration by PGM coated beads (% of spiked norovirus inoculum recovered)	±95 %CI
10% suspension	62%**	1.8	87%	0.6	nvd	n/a
1:10	52%**	1.9	72%	0.4	nvd	n/a
1:100	nvd[1]	n/a[2]	83%	0.1	nvd	n/a
1:1,000	nvd	n/a	85%	1.7	nvd	n/a
1:10,000	nvd	n/a	87%	1.7	nvd	n/a
1:100,000	nvd	n/a	88%	2	nvd	n/a

[1] nvd=no virus detected [2]n/a=not applicable

3.3.2. Capture: determination of the optimal PGM-conjugated bead concentration for norovirus capture

Activated magnetic beads were coated with 0.15mg/ml, 0.38mg/ml and 0.75mg/ml of PGM. Fifty millilitre volumes of PBS containing the PGM conjugated beads were spiked with 200µl of a ten-fold dilution series of norovirus GII. All experiments were conducted in triplicate (Figure 9, Appendix E). The difference in average Cq values at each ten-fold dilution was not significant as the averages remained within 3.3 Ct or 1 log. Activated magnetic beads were conjugated with 0.15mg/ml of PGM in all future experiments as validated; this concentration of PGM was also applied to the capture of norovirus GI strains as demonstrated in section 3.3.4 during the optimisation of pH conditions.

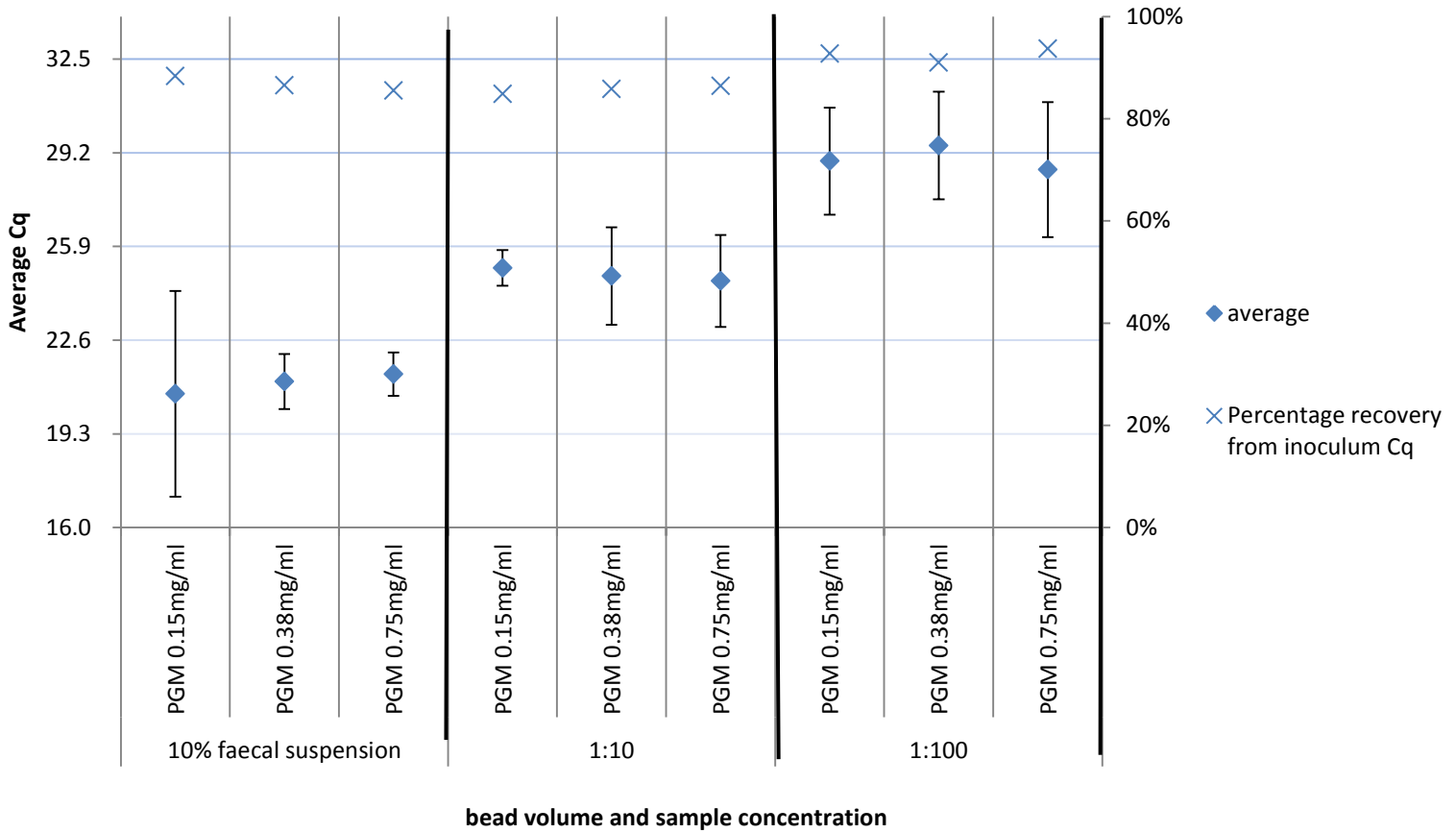


Figure 9 Average Cq value of a ten-fold dilution of norovirus GII captured and detected from 50ml of PBS (pH7) using beads coated with PGM at the following concentrations: 0.15mg/ml, 0.38mg/ml or 0.75mg/ml. Experiments were conducted in triplicate (±95%CI) (Appendix E).

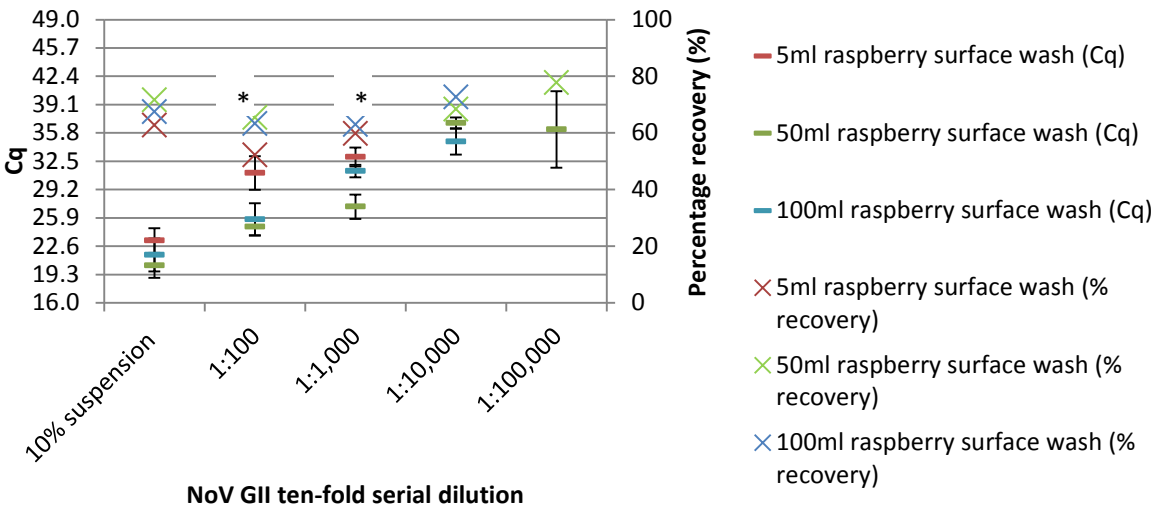
3.3.3. Capture: determining suitable surface wash volume for samples

A larger volume suspension of norovirus GII was prepared, a ten-fold serial dilution of the norovirus GII suspension was then used to artificially contaminate the surface of 25g of raspberries, ham and lettuce (inoculum). The inoculum was added as a single 200µl spot on the food surface, and incubated for 30 seconds in a laminar flow hood. The inoculum was still wet when the contaminated food was washed in a Separator 400 Blender Bag with 5ml, 50ml or 100ml wash volumes of PBS at pH 7 and the entire food surface wash volume was transferred to a 50ml tube, and subjected to PGM capture, nucleic acid extraction and norovirus-real-time RT-PCR (Kageyama *et al.,* 2003). The remaining food in the blender bag was discarded. The foods were artificially contaminated in triplicate for each wash volume at each ten-fold dilution of the norovirus GII inoculum. The norovirus GII strain used for spiking the food samples was selected and applied for consistency, as the evaluation was conducted for the purpose of determining the physical processing of the different wash volumes from different foods. The application of PGM to capture norovirus genotypes GI and GII was demonstrated further in Figure 11.

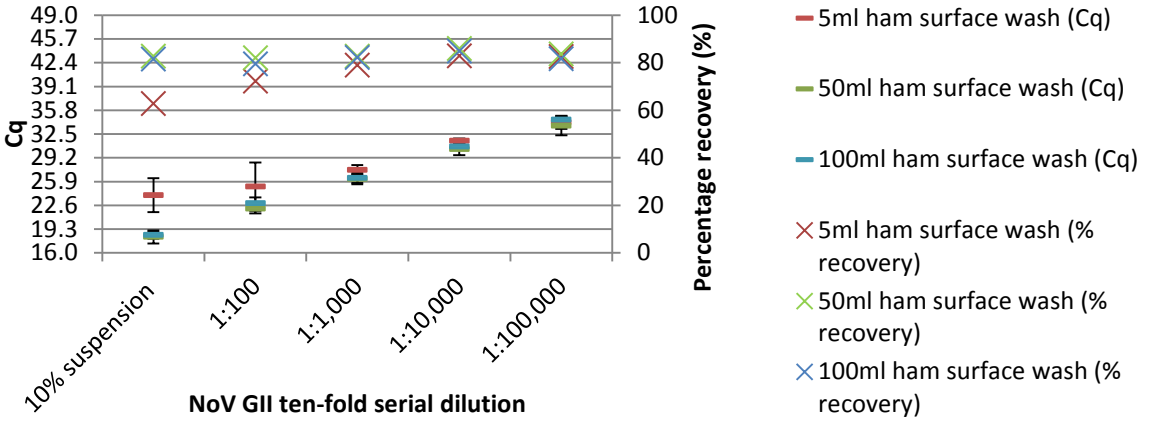
The recovery of norovirus from raspberries using the 5ml surface wash volume was significantly poor based on 95% confidence intervals compared to 50ml and 100ml surface wash volumes when contaminated with the 1:100 and 1:1,000 dilution of the norovirus inoculum. For ham and lettuce the recovery of norovirus inoculum using 5ml, 50ml or 100ml wash volumes was not significant based on 95% confidence intervals (Figure 10). The Cq values varied depending on the food matrices, with highest recovery from the ham samples ranging from 63% to 85% recovery across the dilutions and three wash volumes. There were no significant differences between Cq values obtained with 50ml and 100ml wash volumes based on 95% confidence intervals. However, the Cq values of norovirus

inoculum recovered from 5ml surface wash volumes were significantly poorer in comparison to 50ml and 100ml based on 95% confidence intervals, and failed to be detected at all from raspberries and lettuce contaminated with a 1:100,000 dilution of the inoculum (Figure 10, Appendix F). The 50ml wash volume was large enough to thoroughly wash the entire surface of the RTE food, whilst the 100ml wash volume did not provide any increase in recovery, and sample handling was hampered requiring sample aliquoting to allow subsequent processing, hence 50ml wash volumes were used in all future experiments.

A.



B.



C.

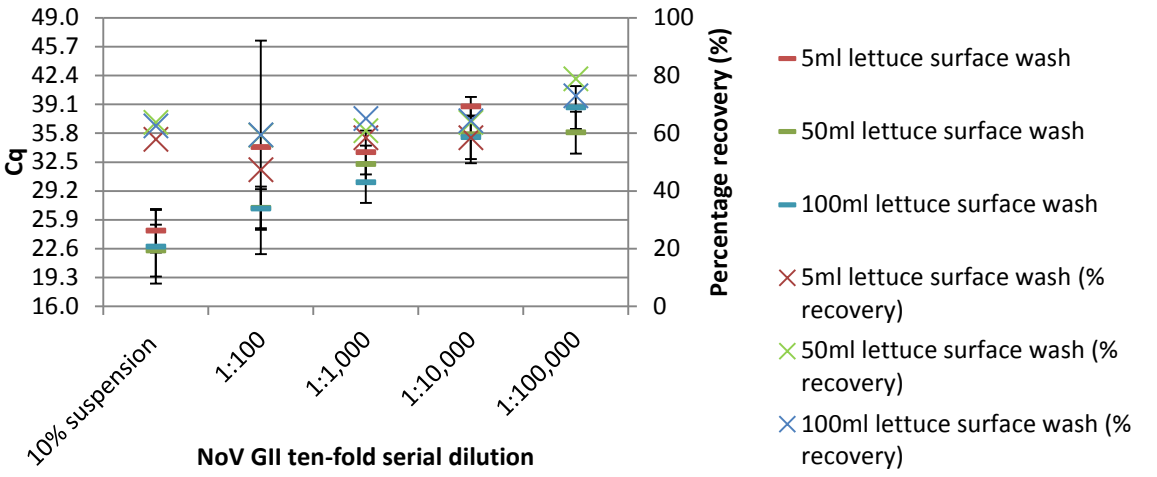


Figure 10 average Cq value of norovirus GII detected from artificially contaminated food, A. raspberries, B. lettuce and C. ham surface washed in three different wash volumes (5ml, 50ml & 100ml) of PBS (pH7), and captured using PGM coated magnetic beads [0.15mg/ml], tested in triplicate (± 95%CI) (Appendix F).

* =statistical significance between sample volumes

3.3.4. Capture: optimisation of pH conditions

Interactions between norovirus and the PGM can be influenced by the environment in which the capture takes place. Specifically, the pH conditions can determine the isoelectric charge of molecules, which may affect the affinity of the interactions between the HBGA and norovirus impacting on the efficiency of the virus capture. Optimal pH conditions for capture of norovirus by PGM were determined by the washing foods at different pH, with consideration for the integrity of norovirus capsids. A 10% suspension of norovirus inoculum was prepared and 200µl was diluted further 1:250 in a 50ml total volume of PBS. The PBS solutions were adjusted to three separate pH levels; pH 3.5, pH 7.0 and pH 10.0. This experiment was undertaken twice; once using a GI norovirus inoculum and once using a GII norovirus inoculum. Each pH was tested in duplicate and repeated twice for both norovirus genogroups. Norovirus GI and GII behaved differently at different pH. Specifically for norovirus GI the greatest recoveries were observed at pH 3.5, whilst recovery decreased with increasing pH, and was significantly reduced at alkaline pH (Figure 11, Appendix G), whereas for norovirus GII recoveries at pH 3.5 and pH 10.0 were comparable.

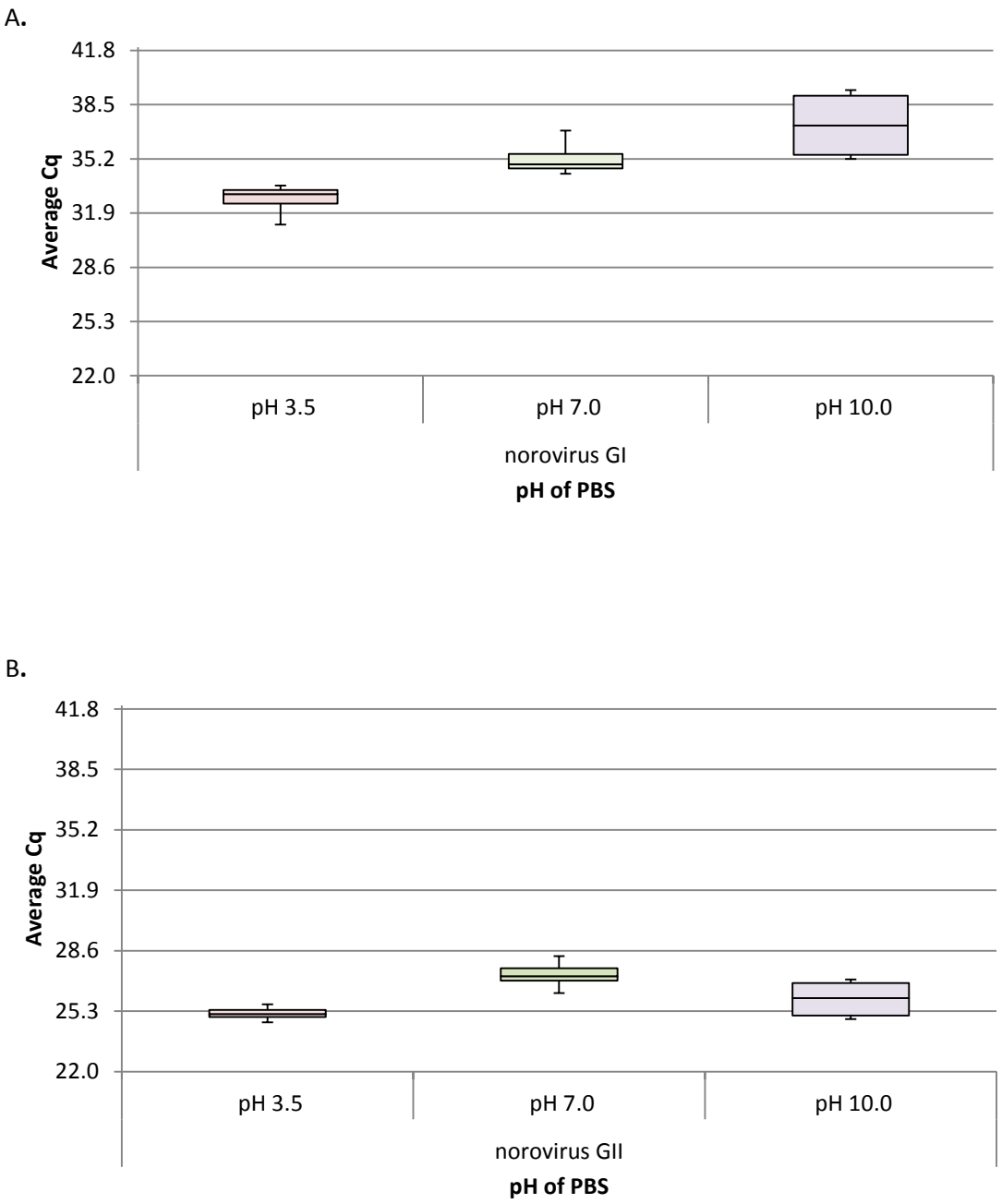


Figure 11 A. Average Cq values of norovirus GI inoculum (pH 7) captured from different pH buffers (pH 3.5-10.0) in 50ml volumes, conducted in duplicate across 2 inter-repeats in a box and whisker plot B. Average Cq values of norovirus GII inoculum (pH 7) captured from different pH buffers (pH 3.5-10.0) at 50ml volumes, conducted in duplicate across 2 inter-repeats in a box and whisker plot Pink = acidic pH, Green= neutral pH, Purple= alkaline pH (Appendix G)

3.3.5. Capture: optimisation of norovirus inoculum captured from 50ml of food surface wash under different pH conditions

Determination of the optimal pH conditions for virus capture by PGM from raspberries and strawberries was undertaken. The natural pH of raspberries (pH 6.8 to pH 6.9) and strawberries (pH 7.1 to pH 7.2) was obtained from surface washing 450g of each fruit in 900ml wash. A total of 300ml of each fruit wash was aliquoted into six replicate 50ml volumes, 300ml was pH adjusted to pH 3.5 and aliquoted into six replicate 50ml volumes, the remaining 300ml was pH adjusted to pH 10.0 and aliquoted into six replicate 50ml volumes. The experiment was replicated with a GI or a GII norovirus.

The average Cq values were lower at pH 3.5 for raspberries and strawberries contaminated with either norovirus GI or GII, however the pH adjustments did not significantly change the efficiency of the capture of norovirus GI (Figure 12 A, Appendix H) and norovirus GII from food washes at pH 3.5 in comparison to the natural pH of food or food washes at pH 10.0 (Figure 12 B, appendix I). However, given the consistent trend for lower Cq values obtained with capture at low pH, all subsequent experiments were conducted at pH 3.5.

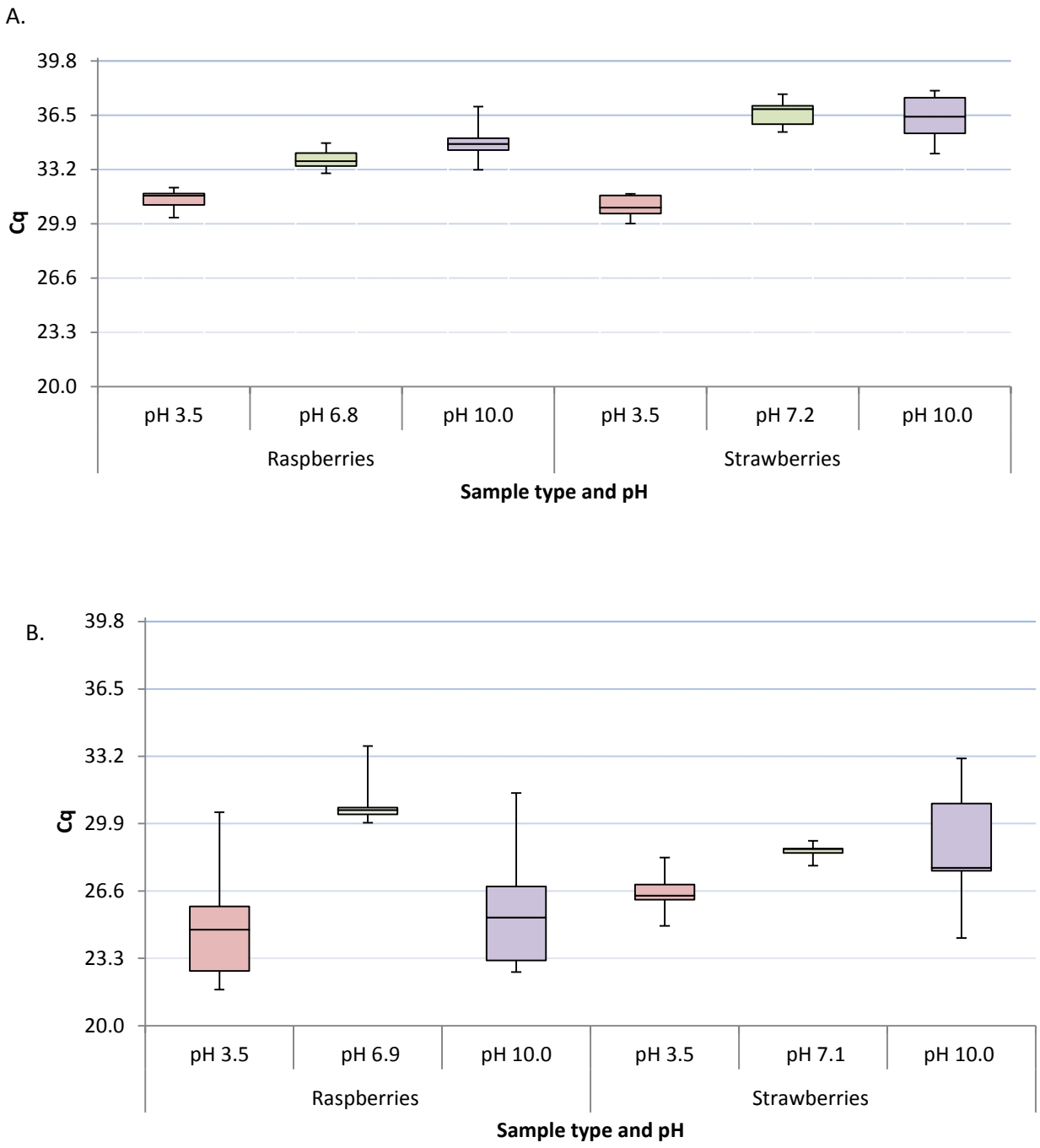


Figure 12 A. Box and whisker plot of norovirus GI captured from raspberries and strawberries washed in 50ml of PBS and left at soft fruit's natural pH (pH 6.8-7.2) compared with raspberries and strawberries washed in 50ml of PBS and adjusted to pH3.5 or adjusted to pH 3.5 or pH 10.0, carried out in triplicate across two inter-repeats (Appendix H). B. Box and whisker plot of norovirus GII captured from raspberries and strawberries washed in 50ml of PBS and left at soft fruit's natural pH (pH 6.9-7.1) compared with raspberries and strawberries washed in 50ml of PBS and adjusted to pH3.5 or adjusted to pH 10.0, carried out in triplicate across two inter-repeats (Appendix I) Pink = acidic pH, Green= neutral pH, Purple= alkaline pH.

3.3.6. Capture: optimisation of incubation temperatures and time

In order to determine optimal incubation conditions for norovirus capture by PGM, a norovirus GII-5 positive 10% faecal suspension was used to artificially contaminate 50ml volumes which were incubated at 4^{o}C, ambient temperature (as defined in Table 3) and 37^{o}C for time intervals of 30, 60, 120 minutes and overnight (Figure 13, Appendix J). The experiment was carried out in duplicate across three sample repeats to give six 50ml replicates at each incubation temperature and time. All Cq values remained within 1 log, therefore no statistically significant differences were observed between incubation temperatures and times. Overnight incubation times for all three incubation temperatures did show an increase in Cq values and a decrease in percentage of norovirus inoculum recovered compared to shorter time periods. Specimens incubated at 37^{o}C overnight resulted in the poorest norovirus recovery. Therefore, overnight incubation and 37^{o}C incubation temperatures were excluded from further validation experiments. Although not statistically significant, the highest recovery was observed at 60 minutes for all three incubation temperatures. As virus capture at 4^{o}C and ambient temperature was consistent, validation experiments were continued at both these incubation temperatures.

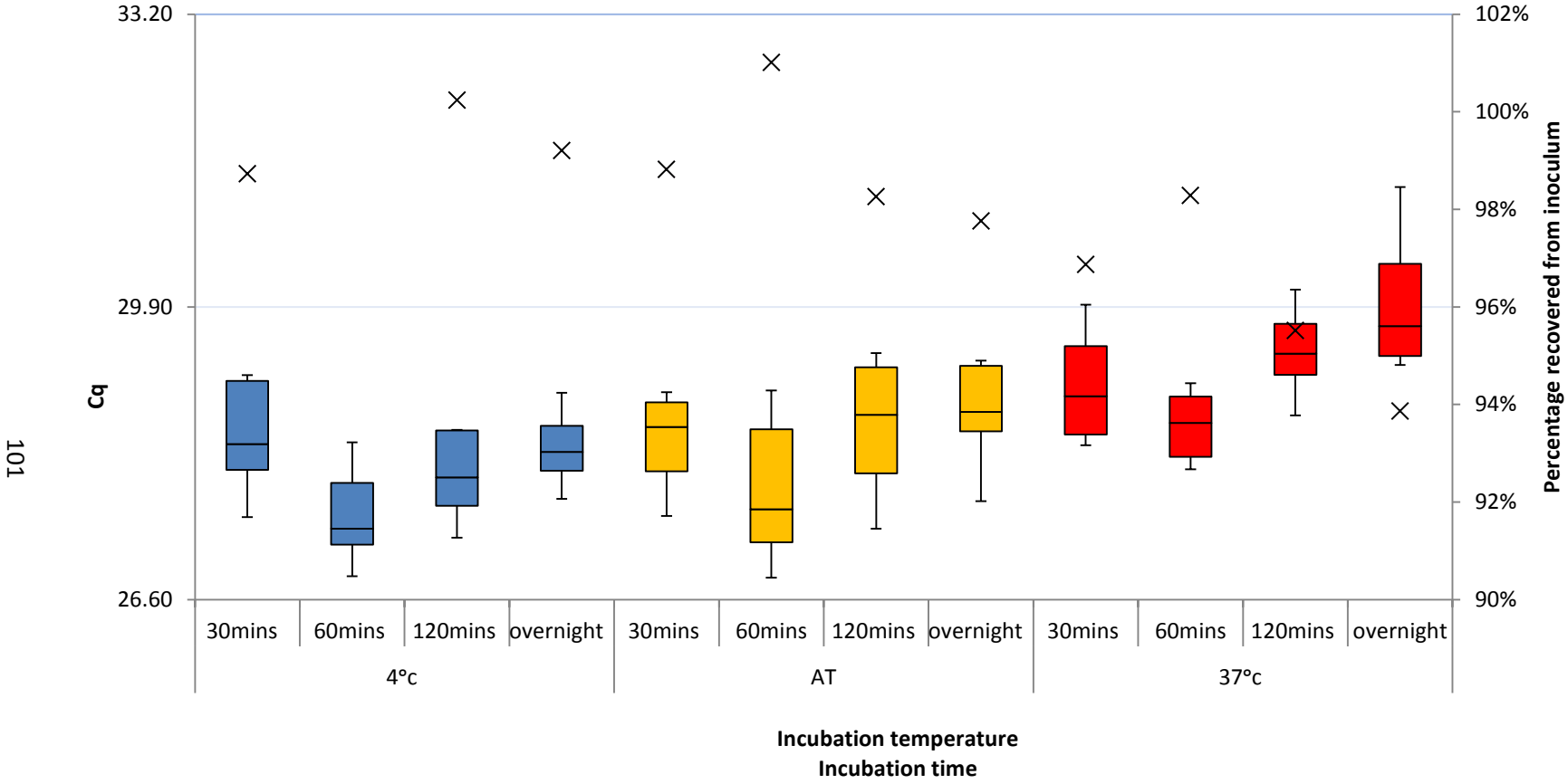


Figure 13 A box and whisker plot of the average Cq of norovirus GII-5 captured from six replicate 50ml specimens of PBS (pH 3.5) incubated at three different temperatures (4°C ambient temperature (AT) and 37 °C tested at 4 different time points (30 minutes, 60 minutes, 120 minutes and overnight).Crosses show the percentage recovered from norovirus inoculum (Appendix J).

3.3.7. Capture: performance of optimised methods with food matrices

Incubation temperatures of 4^{o}C and ambient temperature with a 60 minute incubation time were used to capture norovirus from artificially contaminated foods washed in a 50ml PBS solution adjusted to pH 3.5 (Figure 14, Appendix K). For these experiments, artificial contamination was undertaken with a GII-5 norovirus positive 10% faecal suspension to maintain sample consistency with the previous experiment, so that the optimal incubation temperature from food could be identified. Results were analysed against a norovirus cDNA standard curve generated from validated standard curve material (Appendix L). Greater recovery was achieved for each food type incubated at 4^{o}C. However, the amount of norovirus recovered from specific food types at 4^{o}C or ambient temperature remained within 1 log, and therefore differences were not statistically significant. For these experiments, artificial contamination with an average input inoculum of 2.8 x 10^6 cDNA copies per µl of inoculum was undertaken using a norovirus GII-5 positive 10% faecal suspension. The greatest recovery was achieved for each food type when incubation during capture was conducted at 4^{o}C. For example, 5.8 x 10^5 cDNA copies per 25g of strawberries was recovered, 4.7 x 10^5 cDNA copies per 25g of lettuce was recovered and 6.2 x 10^5 cDNA copies per 25g of ham was recovered at 4^{o}C compared to 1.8 x 10^5 cDNA copies per 25g of strawberries was recovered, 3.2 x 10^5 cDNA copies per 25g of lettuce was recovered and 3.3 x 10^5 cDNA copies per 25g of ham incubated at ambient temperature. The data was consolidated into a summary table (Table 17) and a greater percentage of norovirus recovery was observed from strawberries (21%), lettuce (17%) and ham (23%) when incubated at 4^{o}C compared to the percentage recovery of norovirus from strawberries (7%), lettuce (12%) and ham (12%) when incubated at ambient temperature. Overall the amount of norovirus recovered from all food products incubated at 4^{o}C was greater

compared to ambient temperature it was decided to undertake all further validation work at 4^{o}C.

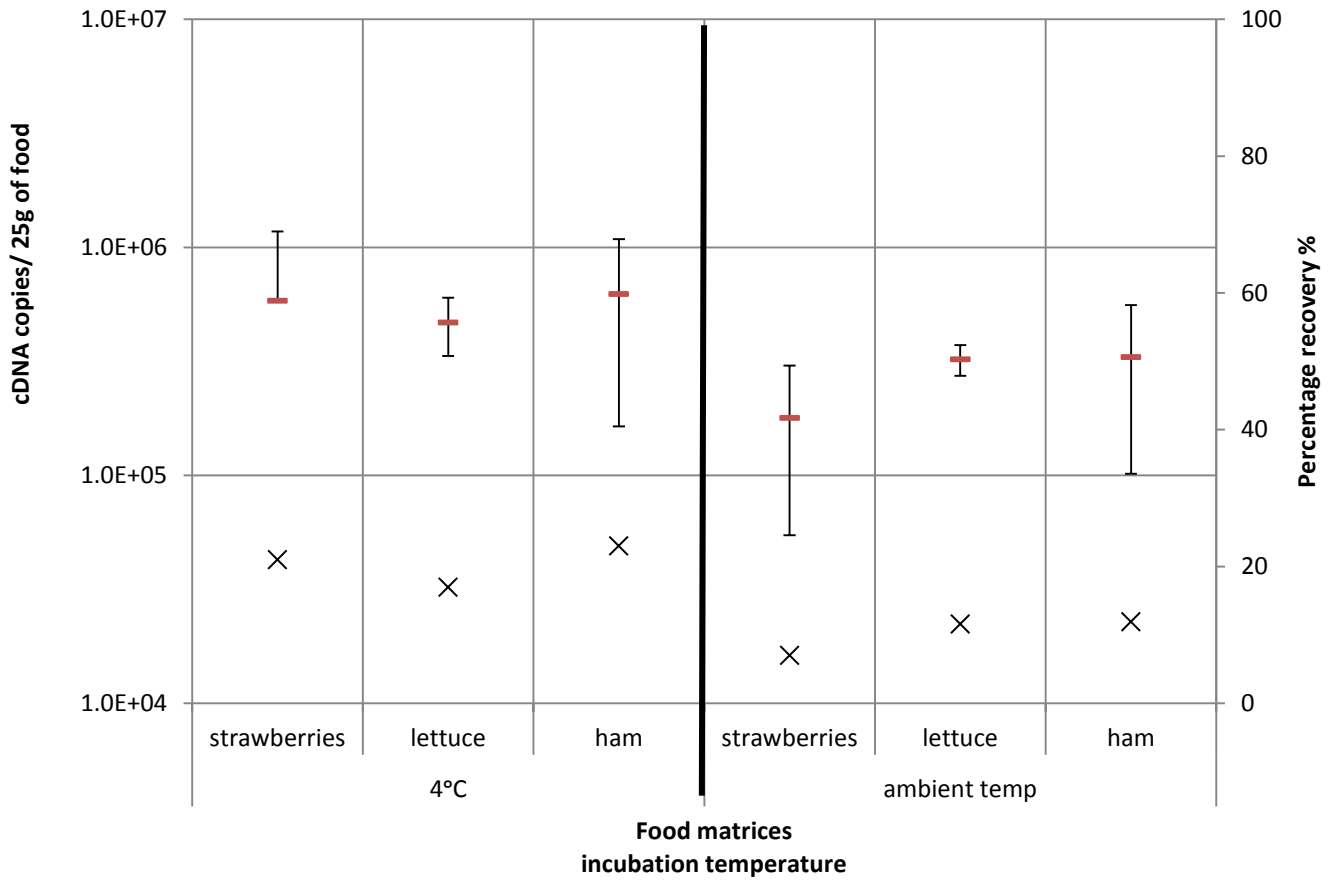


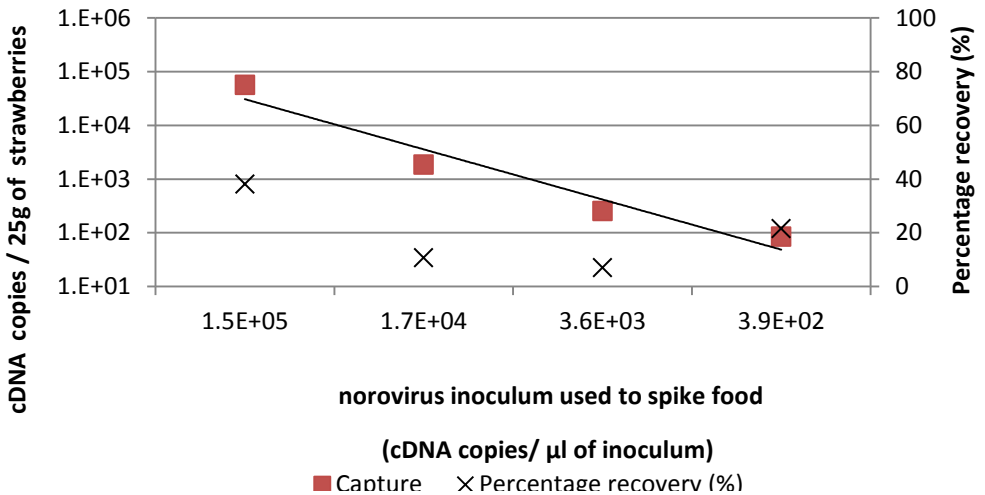
Figure 14 Average cDNA copies of norovirus GII-5 detected per 25g of strawberries, lettuce and ham washed in PBS (pH 3.5) and incubated at 4^{o}C or Ambient temperature (AT) for 60 minutes carried out in duplicate. Error bars= (±SD) (Appendix K).

Table 17 A summary table showing the average cDNA copies of norovirus GII-5 recovered from 25g of strawberries, lettuce and ham when the sample was incubated at either 4^0C or ambient temperature and the percentage of norovirus recovered. Food was tested in triplicate.

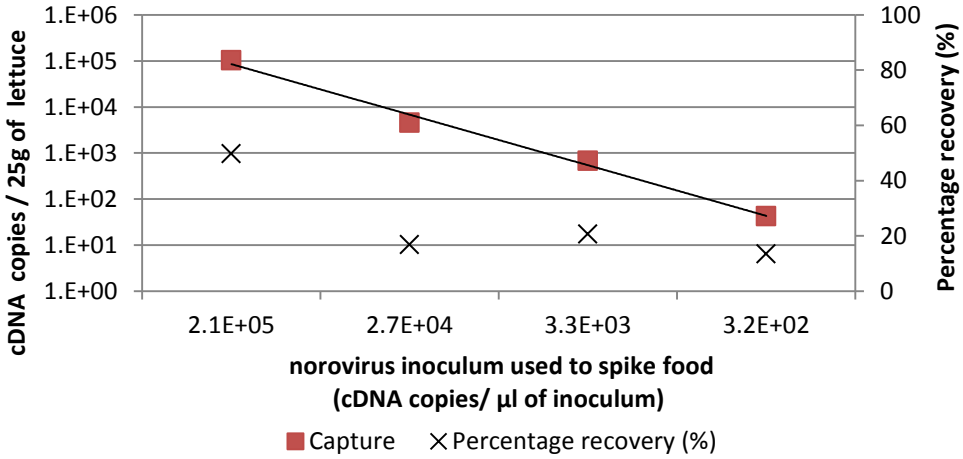
Incubation temperature	Food type	average input cDNA copies per µl of inoculum used to contaminate food ± SD	average cDNA copies per 25g of food detected ± SD	Percentage recovered (average input cDNA copies per µl of inoculum / average cDNA copies per 25g of food detected x100)
4^0c	Strawberries	$2.8 \times 10^6 \pm 9.5 \times 10^4$	$5.8 \times 10^5 \pm 5.9 \times 10^5$	21%
	Lettuce		$4.7 \times 10^5 \pm 1.3 \times 10^5$	17%
	Ham		$6.2 \times 10^5 \pm 4.6 \times 10^5$	23%
Ambient temperature	Strawberries		$1.8 \times 10^5 \pm 1.2 \times 10^5$	7%
	Lettuce		$3.2 \times 10^5 \pm 4.9 \times 10^4$	12%
	Ham		$3.3x\ 10^5 \pm 2.3 \times 10^5$	12%

A ten-fold dilution series of a norovirus GII-5 inoculum was used to artificially contaminate the surface of strawberries, lettuce and ham and the number of cDNA copies recovered was calculated in order to determine the sensitivity of detection from these food types (Figure 15). The food surfaces were washed in 50ml of PBS (pH 3.5) and incubated with PGM magnetic beads for 60 minutes at 4^{o}C and tested in triplicate. The limit of detection was 84 cDNA copies per 25g of strawberries, 43 cDNA copies per 25g of lettuce and 120 cDNA copies per 25g of ham. The average number of norovirus cDNA copies detected per 25g of ham was higher compared to strawberries and lettuce. The data was consolidated in a summary (Table 18) and the recovery of norovirus from ham was better, despite the inoculum used to artificially contaminate the ham was the lowest out of the three food types. Ham was artificially contaminated with 2.6×10^2 cDNA copies per µl of inoculum**,** strawberries were contaminated with 3.9×10^2 cDNA copies per µl of inoculum and lettuce with 3.2×10^2 cDNA copies per µl of inoculum. The percentage of norovirus recovered was greatest from ham at 47% followed by strawberries at 23% and lettuce at 13%.

A.



B.



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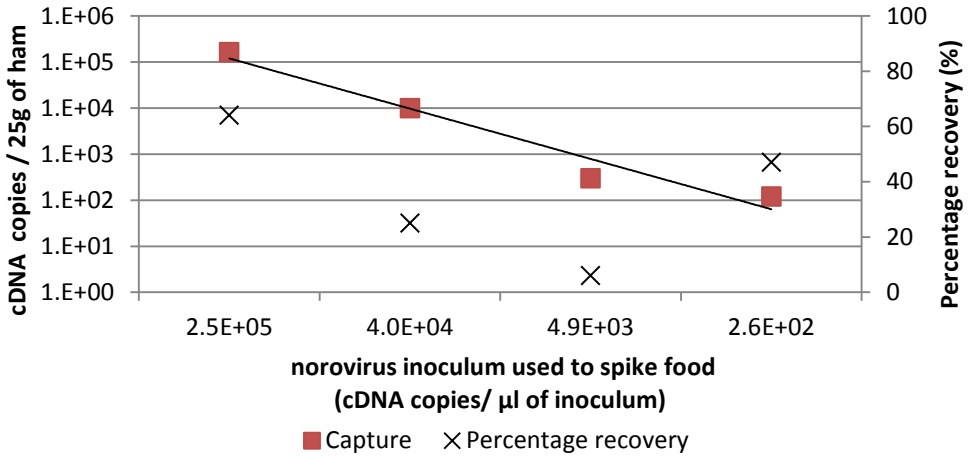


Figure 15 The average number of norovirus GII-5 cDNA copies/ 25g of artificially contaminated foods A. strawberries B. lettuce C. ham applied by pipetting 200µl of inoculum under optimal conditions carried out in triplicate. Crosses show the percentage of norovirus recovered from artificially contaminated food based on the input inoculum at each dilution.

Table 18 A summary table showing the limit of detection of norovirus GII-5 from strawberries, lettuce and ham from the average input cDNA copies per µl of inoculum used to artificially contaminate the food, the average cDNA copies per 25g of food detected and the percentage recovered

Food type	average input cDNA copies per µl of inoculum used to contaminate food ± SD	average cDNA copies per 25g of food detected ± SD	Percentage recovered (average input cDNA copies per µl of inoculum / average cDNA copies per 25g of food detected x100)
Strawberries	$3.9 \times 10^2 \pm 2.9 \times 10^2$	$8.5 \times 10^1 \pm 6.2 \times 10^0$	23%
Lettuce	$3.2 \times 10^2 \pm 2.9 \times 10^2$	$4.3 \times 10^1 \pm 5.3 \times 10^1$	13%
Ham	$2.6 \times 10^2 \pm 1.4 \times 10^2$	$1.2 \times 10^2 \pm 6.7 \times 10^1$	47%

3.4. Nucleic acid extraction optimisation

3.4.1. Extraction: assessment of automated extraction platforms specimens

A comparison of four different automated nucleic acid extraction platforms were compared using a 10% norovirus faecal suspension, as validation data specifically for foods had not been published previously by any of the manufacturers, and the platforms had been designed with extraction of genomic material from clinical specimens in mind. Using a norovirus GII positive faecal specimen, the aim was to identify the sensitivity of each platform from a dilution series of the faecal sample, each dilution represented as four replicates. The platforms evaluated (Promega Maxwell 16™, the QIAgen QIAxtractor™, the QIAgen QIAsymphony™ and the Roche MagNA Pure 96™) had different throughput capabilities which were considered for the development of a high throughput protocol for the extraction of viral nucleic acids from food matrices for use in food laboratories (Table 19).

Table 19 List of automated extraction platforms assessed showing the total number of samples that can be processed and the time taken for the extraction to complete.

	Roche Mag*NA* Pure 96™	QIAgen QIAymphony™	QIAgen QIAxtractor™	Promega Maxwell 16™
Throughput number	96 samples	96 samples	96 samples	16 samples
Extraction time for a complete run	1 hour	3 hours 52 minutes	1 hour 30 minutes	45 minutes
Input volume (+ lysis buffer if required)	200µl to 1ml	630µl	100µl to 300µl	600µl
Output volume	110µl to 200µl	60µl to 190 µl	100µl to 200µl	100µl plus
Cost per sample for consumables[1]	£11.28	£7.33	£7.20	£7.16
Labour time	20 minutes	30 minutes	30 minutes	30 minutes
Continuous or batch processing	Batch 96 samples	Batch 96 samples or continuous 24 samples	Batch 96 samples	Batch 16 samples
Technology	Magnetic beads	Magnetic beads	Filtration	Magnetic beads

[1]prices correct at September 2016

Nucleic acid from the norovirus GII positive faecal specimens was extracted using the Promega Maxwell 16™ Total RNA Purification Small Elution Volume kit, the QIAgen QIAxtractor™ Virus Plasticware Kit, the QIAgen QIAsymphony™ Virus/Pathogen Mini kit or the Roche Mag*NA* Pure 96™ DNA and viral RNA small volume kit in initial experiments (Figure 16, Appendix M). A single faecal sample containing norovirus GII was serially diluted and selected for extraction to maintain sample consistency throughout the comparison of the four automated extraction platforms. The QIAgen QIAsymphony™ and the Roche Mag*NA* Pure 96™ extraction methods resulted in a 10 fold increase in sensitivity for the detection of norovirus compared to the Promega Maxwell 96™ and QIAgen QIAxtractor™ extraction platforms. Mengovirus internal process control was also consistently detected by the QIAgen QIAsymphony™, QIAgen QIAxtractor™ and the Roche Mag*NA* Pure 96™ platforms across the dilution series. Mengovirus failed to be

detected consistently by the Promega Maxwell 96™ platform indicating that the performance of the extraction was suboptimal compared to the other platforms (Appendix M). Therefore, the Promega Maxwell 96™ and QIAgen QIAxtractor™ were not considered in further evaluations. The sensitivity of the QIAgen QIAsymphony™ and the Roche Mag*NA* Pure 96 was assessed further in a checkerboard containing 288 specimens comprised of either minimum essential media (MEM) as a negative control, and a ten-fold dilution series of a norovirus GII positive faecal specimen. Both methods were equally sensitive in performance; any difference in detection was not greater than 3.3 Ct and therefore not significant (Appendix N). As these platforms were most sensitive for extracting total nucleic acid from faeces, a protocol for extracting total nucleic acid from faecally contaminated food was designed and evaluated.

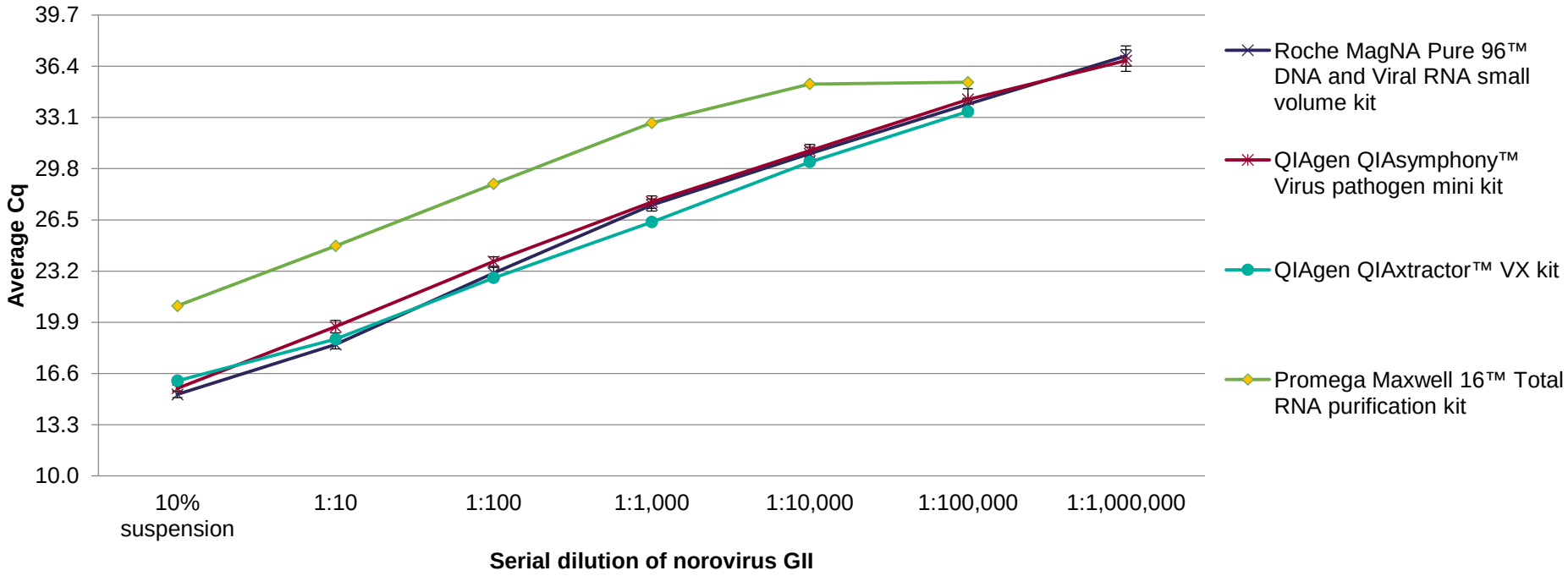


Figure 16 A graph comparing the sensitivity, based on average Cq values of four replicates from the four automated extraction platforms: The Promega Maxwell 16™, the QIAgen QIAxtractor™ the QIAgen QIAsymphony™ and the Roche Mag*NA* Pure 96™. The experiment used norovirus GII inoculum from a ten-fold dilution series ± SD (Appendix M).

As validation data specifically for foods had not been published previously by either of the manufacturers, a protocol for the extraction of viral nucleic acid from faecal contaminated food matrices was adapted for both platforms. For the Roche Mag*Na* Pure 96™ extraction platform, a more costly transition to the DNA and viral RNA large volume kit was required to process food specimens using the Pathogen Universal 500 protocol and consumables. This kit used 300µl of lysis buffer and 200µl of sample. The method was evaluated by extracting three samples types: a ten-fold dilution series of a norovirus GII positive faecal suspension; the same norovirus positive faecal suspension diluted in 50ml volumes and captured by PGM magnetic beads; and raspberries artificially contaminated with the norovirus positive faecal suspension, surface washed and captured by PGM magnetic beads. All samples were extracted four times, and norovirus RNA was detected by real time RT-PCR. There was a significant loss in sensitivity with the Roche Mag*NA* Pure 96 across the dilution series (Figure 17), and no detection of norovirus RNA from the artificially contaminated PGM capture concentrated raspberry washes.

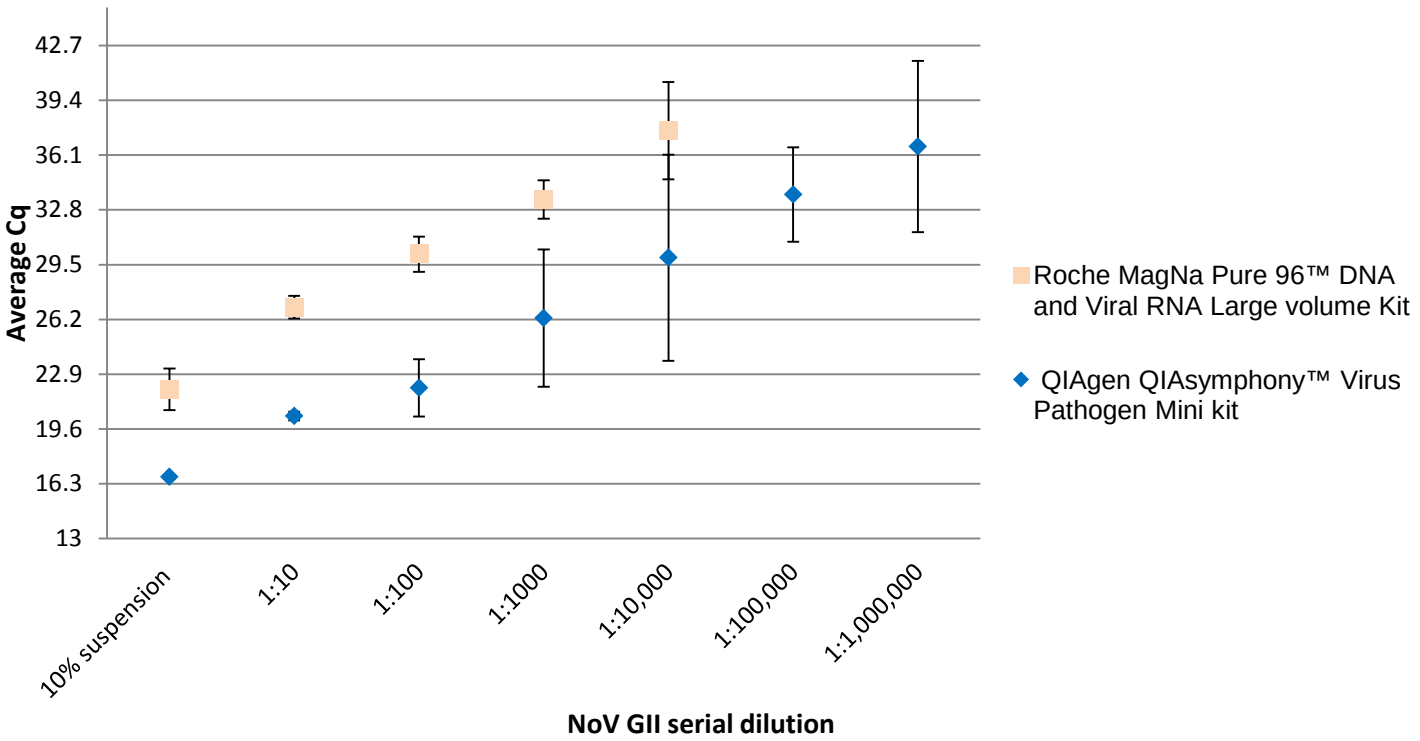


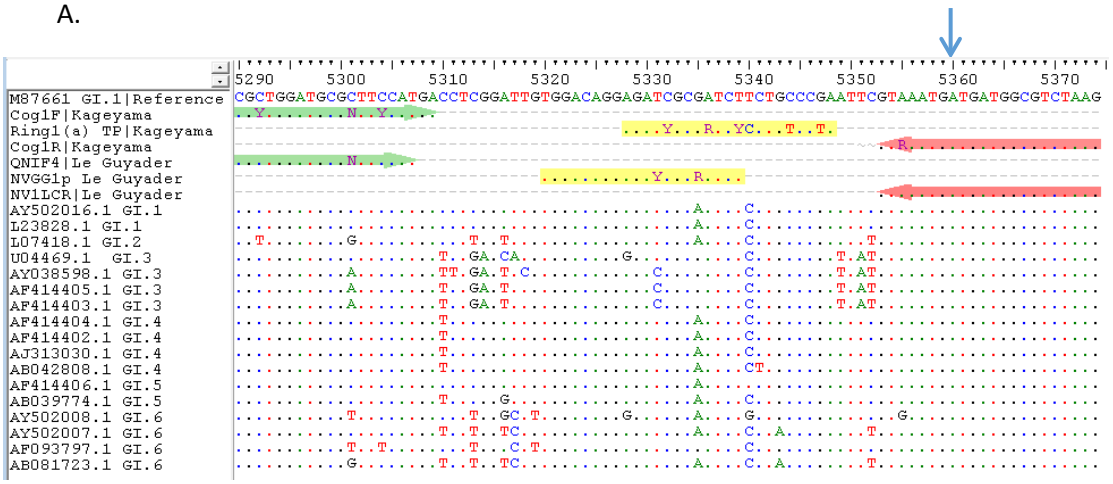
Figure 17 A figure presenting average Cq values of norovirus GII prepared as a ten-fold dilution series from a 10% faecal suspension, then diluted further 1:250 in 50ml volumes of PBS (pH 3.5), norovirus captured by PGM and extracted using the DNA and Viral RNA Large volume kit on the Roche Mag*Na* Pure 96 (pink square) or extracted with QIAgen QIAsymphony Virus pathogen Mini Kit. All experiments were conducted four times. Error bars ±95%CI

3.5. Optimisation of norovirus target detection

3.5.1. PCR: Assessment of sensitivity of two PCR assays for the quantification of norovirus GI and GII using dsDNA standards curves

The primer and probe sets from two previously published PCR assays; the Le Guyader *et al.,* (2009) and the Kageyama *et al.,* (2003) assays were compared in order to identify a PCR method which is sensitive and able to detect norovirus from foods where viral loads are low. The primer sets of both assays were mapped to reference sequences to compare their location and cross reactivity to different genotypes. Degeneracies were present in both primer sets to allow priming of different norovirus GI (Figure 18 A.) and GII genotypes (Figure 18 B.) (Zheng *et al.,* 2006). More degeneracies were present in the Cog1F and Cog1R primer sets (Kageyama *et al.,* 2003) than the QNIF4 and NV1LCR primer sets (Le Guyader *et al.,* 2006) for the detection of norovirus GI. For the detection of norovirus GII, degeneracies were only present in the QNIF2 forward primer (Le Guyader *et al.,* 2006) no other degeneracies were present.

A.



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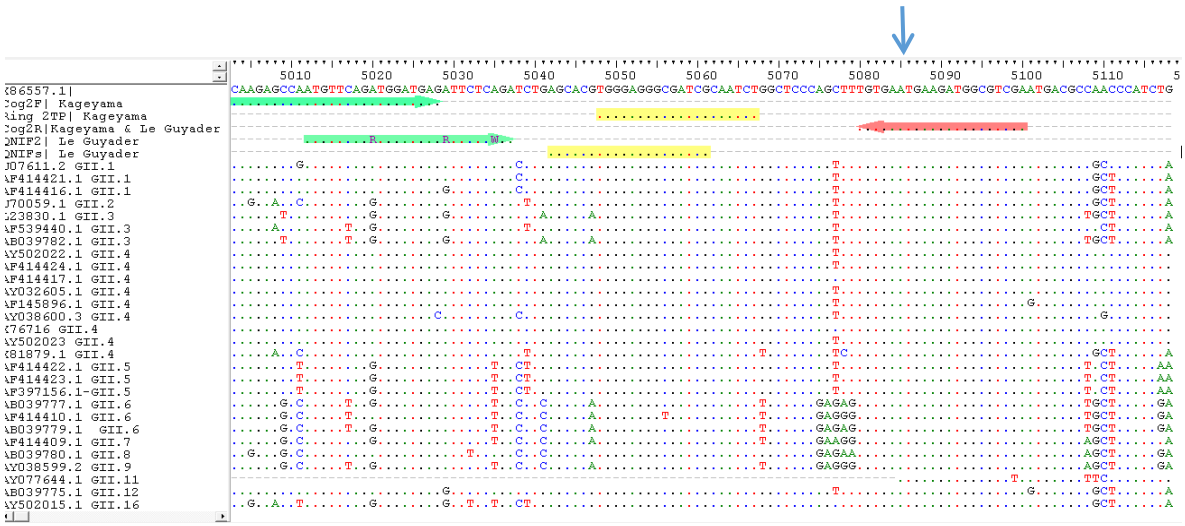


Figure 18 All primer and probe combinations used in PCR analysis. A. norovirus genogroup I detection, mapped against reference whole genome of reference strain GI-I from Zheng *et al.*, (2006) (GenBank accession number M87661. B. norovirus genogroup II detection reference strain GII-4 (GenBank accession number X86557.1) to demonstrate cross reactivity with different entries of norovirus genomes in GenBank (GenBank Accession number and norovirus genotypes). Dark green arrow= forward primer, yellow= probe, red arrow= reverse primer) dots= matching nucleotides, degeneracies Y=C or T, N=A, C,T,G, R=G or A, W=A or T. Blue arrow above ruler= start of ORF 2

Standard curves were generated for norovirus GI and GII using the dsDNA plasmid standards as described in ISO/TS 15216 (2013). The standards were a ten-fold dilution series containing 10 to 100,000 dsDNA copies per µl, and average Cq values were generated from two inter-repeats. The standard curves for both assays were comparable as demonstrated by the R^2 values for both norovirus GI and GII (Le Guyader R^2=0.99, Kageyama R^2=0.98). The primer sets for both norovirus GI and GII assays were inter-exchanged to assess their performance characteristics further and select the most robust, broadly reactive and sensitive primer-probe assay format. Differences between the two assays for detection of norovirus GI and GII was not greater than 3.3 Ct and therefore not significant, furthermore, both assays had the same detection limit (Appendix L). All work was conducted using these plasmid standards to quantitate and normalise norovirus detection from food contamination and food handling experiments.

3.5.2. PCR: assay assessment using faecal specimens

The two published PCR assays were compared and evaluated for the detection of four different norovirus genotypes from four different faecal inoculums in 50ml. For each of the four faecal inoculums, a ten-fold dilution series was prepared, starting with a 10% suspension containing 2 x 10^{-2} g of faeces through to the last dilution containing 2 x 10^{-6} g of faeces. These 50ml samples were concentrated using the PGM coated magnetic beads, total nucleic acid captured on the magnetic beads was extracted, and the RNA was split so that cDNA was generated and quantified by the Le Guyader real time RTqPCR assay and by the Kageyama real time RTqPCR assay. All experiments were conducted in triplicate. Data was normalised against a standard curve and expressed as cDNA copies per gram of faeces, as described in section 2.6.3. The average cDNA copies

per gram of faeces were calculated for each genotype from the three replicates at each dilution (Figure 19). The average of each dilution was then used to calculate the overall average amount of cDNA copies per gram of faeces recovered. Average $\log_{10}$ cDNA copies of norovirus GII-4, GII-5 and GI-7 per gram of faeces were similar for both assays as standard error bars of the average $\log_{10}$ cDNA copies of norovirus GII-4, GII-5 and GI-7 overlapped and P values were greater than 0.05 (Appendix O). However the difference between the average $\log_{10}$ cDNA copies in the two assay for the detection of norovirus GII-6 was statistically significant (P=0.004, p=<0.05) (Appendix O).

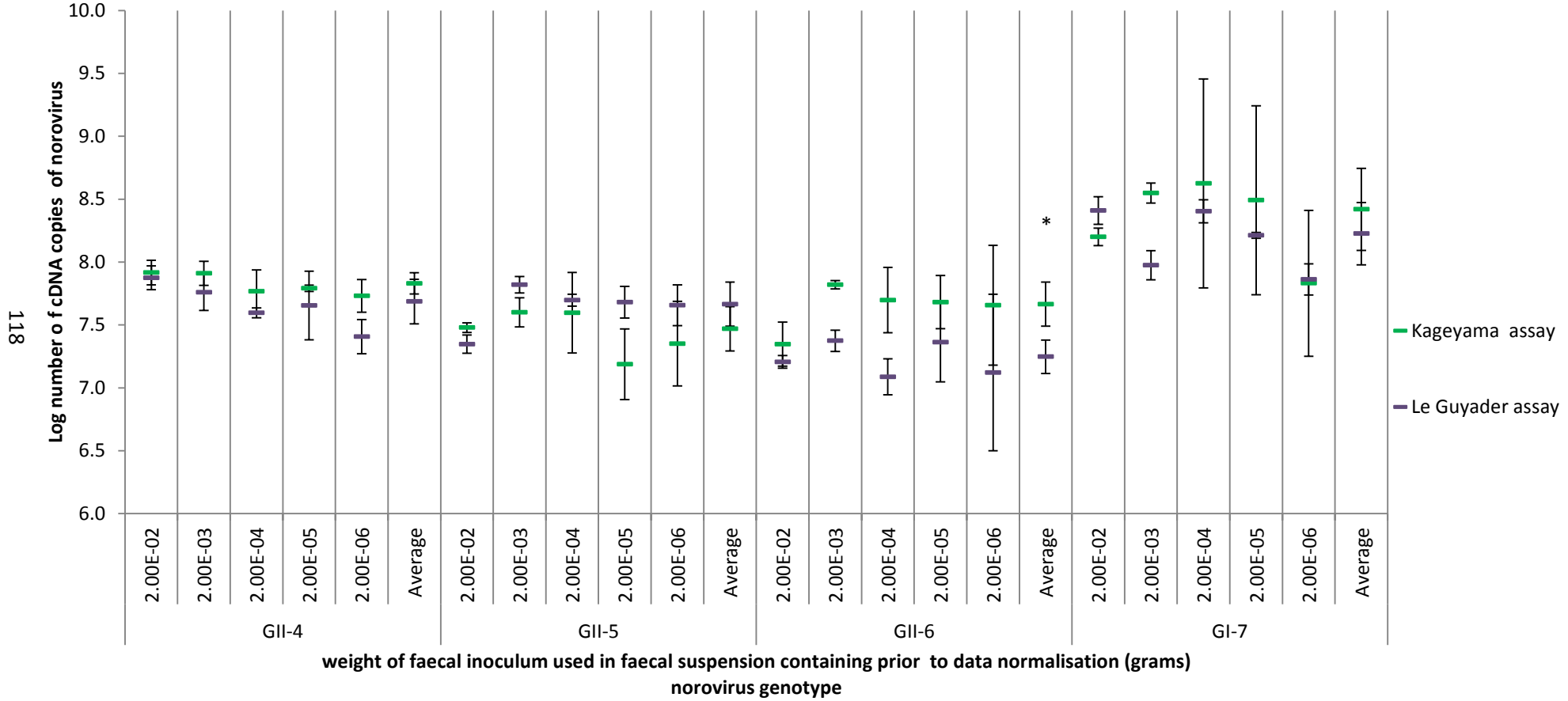


Figure 19 average number of $\log_{10}$ cDNA copies/ gram of faeces in inoculum from three replicates A=derived from cDNA copies/ tNA extract from 2 $x10^{-2}$ g of contaminating inoculum, B= derived from copies/µl inoculum from $2x10^{-3}$ g of contaminating inoculum, C= derived from cDNA copies/ µl inoculum from $2x10^{-4}$ g of contaminating inoculum, D= derived from copies/µl inoculum from $2x10^{-5}$ g contaminating inoculum, E= derived from cDNA copies/ µl inoculum from $2x10^{-6}$ g of contaminating inoculum (green=Kageyama, purple= Le Guyader assay) Error bars= SD. *= statistically significant.

3.5.3. PCR: assay sensitivity

The two PCR assays were subsequently evaluated for the recovery of norovirus from artificially contaminated food matrices under optimised conditions, surface washing the food in PBS (pH 3.5) with PGM magnetic beads to capture nucleic acid incubated in samples at 4^{o}C for 60 minutes. The nucleic acid extraction was performed using the QIAgen QIAsymphony™ platform and complex 200 protocol. The nucleic acid eluate was split so that half the norovirus RNA was quantified by the Le Guyader real time RTqPCR assay and the other half was quantified by the Kageyama real time RTqPCR assay. All standard curves R^2 values ranged between 0.997 and 1.000 (Appendix P). All experiments were conducted in triplicate and presented for both PCR assays to determine which assay was most sensitive at detecting norovirus from food matrices. For these experiments strawberries, lettuce and ham were contaminated with GII-5. In addition ham was contaminated with GII-4, GII-6 and GI-7 as this food type was a RTE food not included in ISO 15216 (2013).

All quantification data was normalised and presented on a $\log_{10}$ scale. The average number of $\log_{10}$ cDNA copies detected from three artificially contaminated food matrices; strawberries (Figure 20), lettuce (Figure 21) and ham (Figure 22) were compared to the average number of $\log_{10}$ cDNA copies/µl of inoculum. The average number of $\log_{10}$ cDNA copies from ham only was then used to compare recovery of three different norovirus genotypes GII-4 (Figure 23), GII-6 (Figure 24) and GI-7 (Figure 25). The amount of $\log_{10}$ cDNA copies/µl of inoculum used to artificially contaminate the food was calculated from validated standard curve material. The $\log_{10}$ cDNA copies/µl of inoculum differed between assays, therefore in order to compare the

performance of the assays, the percentage of norovirus recovered from 25g of strawberries, lettuce or ham was calculated using the following calculation:

$$\frac{\textit{Number of cDNA copies/µl of inoculum detected from food}}{\textit{Number of cDNA copies/µl of input inoculum}} \times 100$$

Although Le Guyader was able to detect more $\log_{10}$ cDNA copies/25g of food contaminated with higher concentrations of norovirus, the percentage recovery was calculated from the lowest concentration of norovirus contamination to identify the assay able to detect the most norovirus from weakly contaminated samples, as expected from naturally contaminated foods. The limit of detection of norovirus cDNA copies in Figure 20 to Figure 25 was consolidated in Table 20. The Kageyama assay showed the greatest percentage recovery of norovirus GII-5 from strawberries at 23% compared to the Le Guyader assay from which recovery of norovirus from strawberries was 12%. The Kageyama assay also showed the greatest recovery from norovirus GII-4 (73%), GII-5 (47%), GII-6 (16%) and GI-7 (84%) from ham compared to the Le Guyader assay from norovirus GII-4 (40%), GII-5 (24%), GII-6 (4%) and GI-7 (24%) from ham. The percentage of norovirus recovered using the Le Guyader assay was higher from lettuce at 84% compared to the Kageyama assay at 13%. Given the assays comparability but better percentage recovery, with the exception of GII-5 from lettuce, which is not one of the most widely distributed genotypes of norovirus; subsequent application of the method to a range of RTE foods was conducted using the Kageyama PCR method.

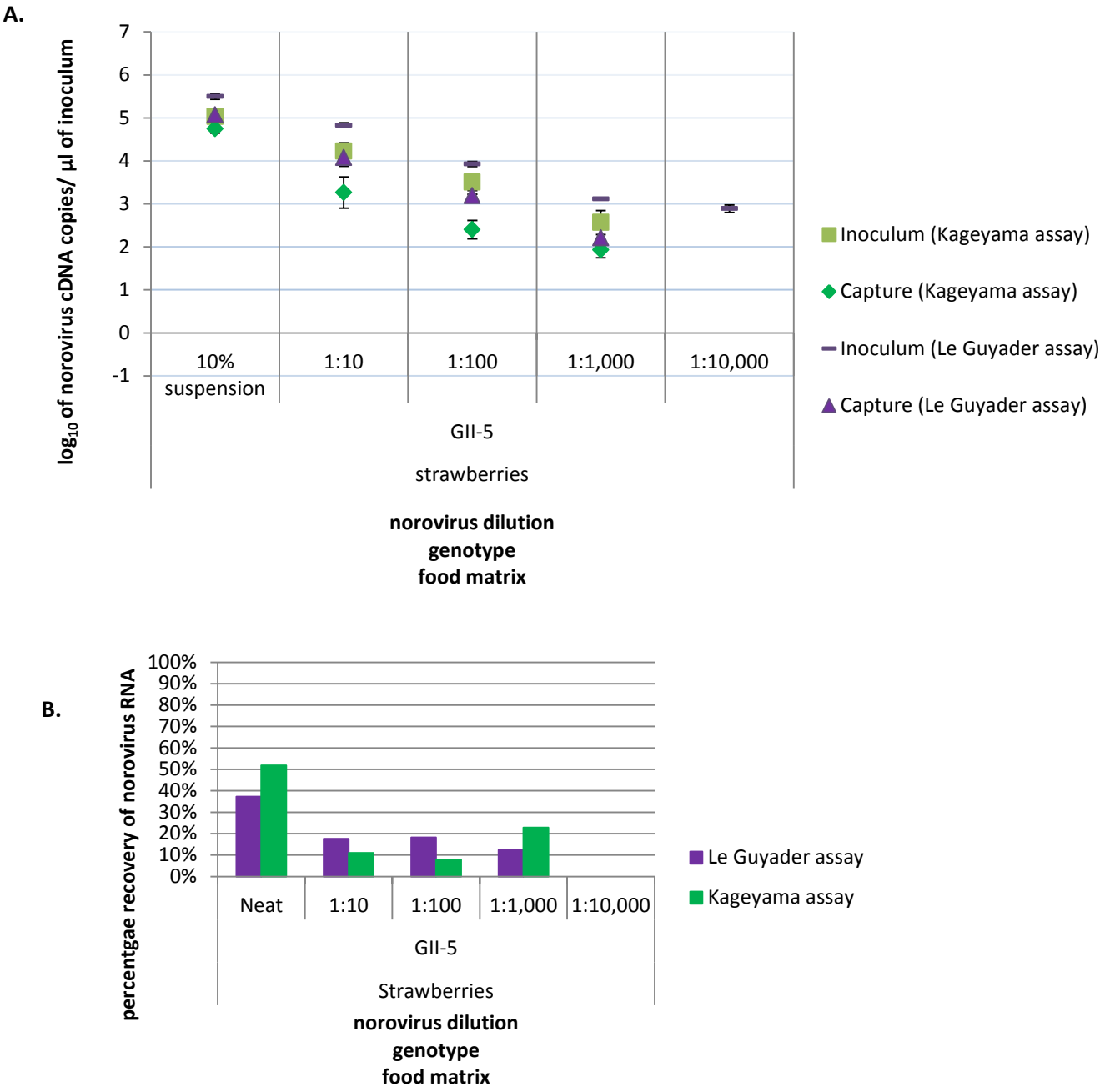


Figure 20 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GII-5 captured from artificially contaminated strawberries by PCR detection using the Le Guyader or the Kageyama PCR assay. Kageyama inoculum and capture, Le Guyader capture was not detected at a dilution of 1:10,000. Error bars=SD. B. figure representing the percentage recovery on the ham from inoculum using the Le Guyader or the Kageyama PCR

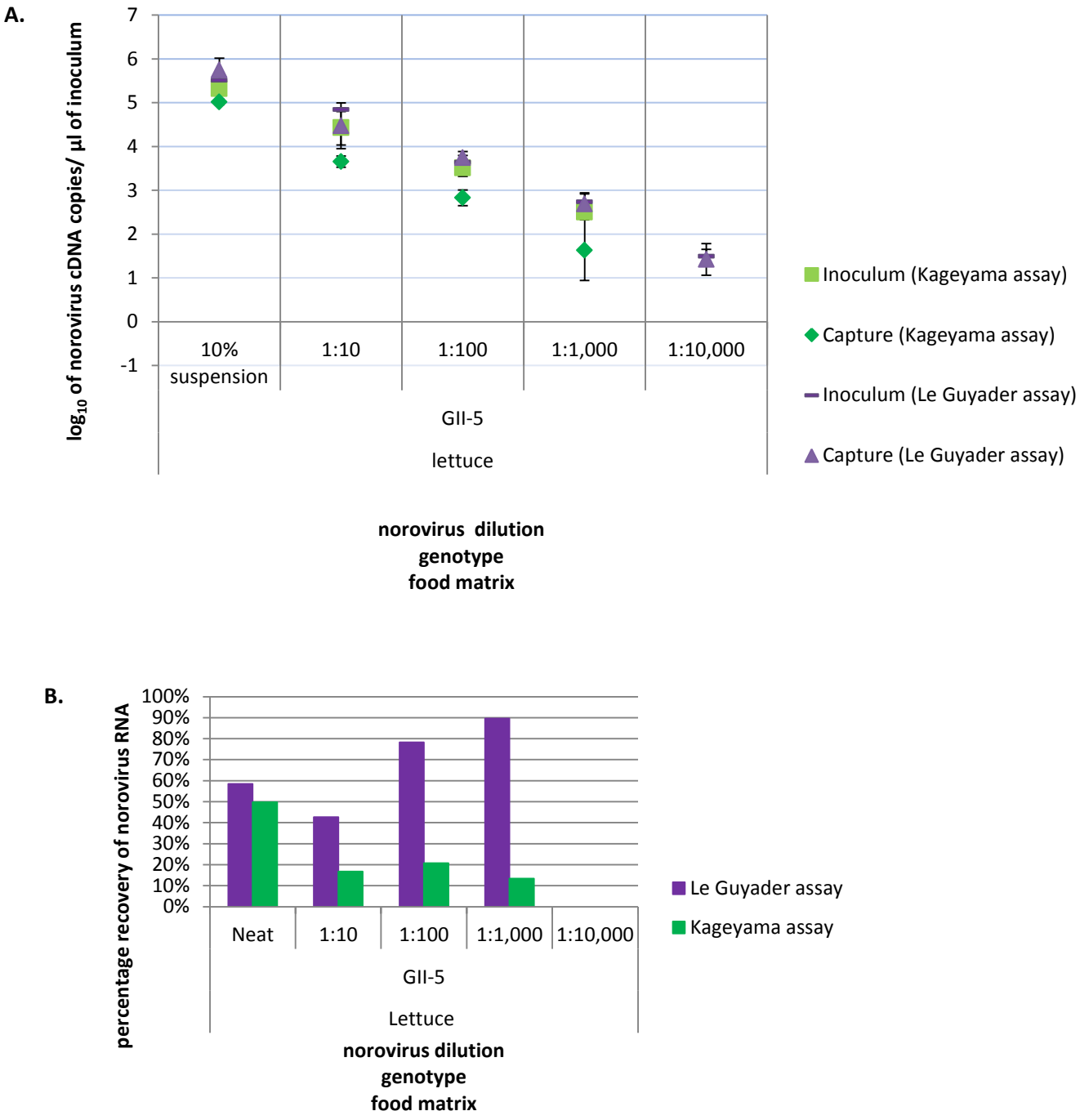


Figure 21 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GII-5 captured from artificially contaminated ham by PCR detection using the Le Guyader or the Kageyama PCR assay. Error bars=SD. Kageyama inoculum and capture not detected at 1: 10,000 dilution. B. figure representing the percentage recovery on the lettuce from inoculum using the Le Guyader or the Kageyama PCR assay.

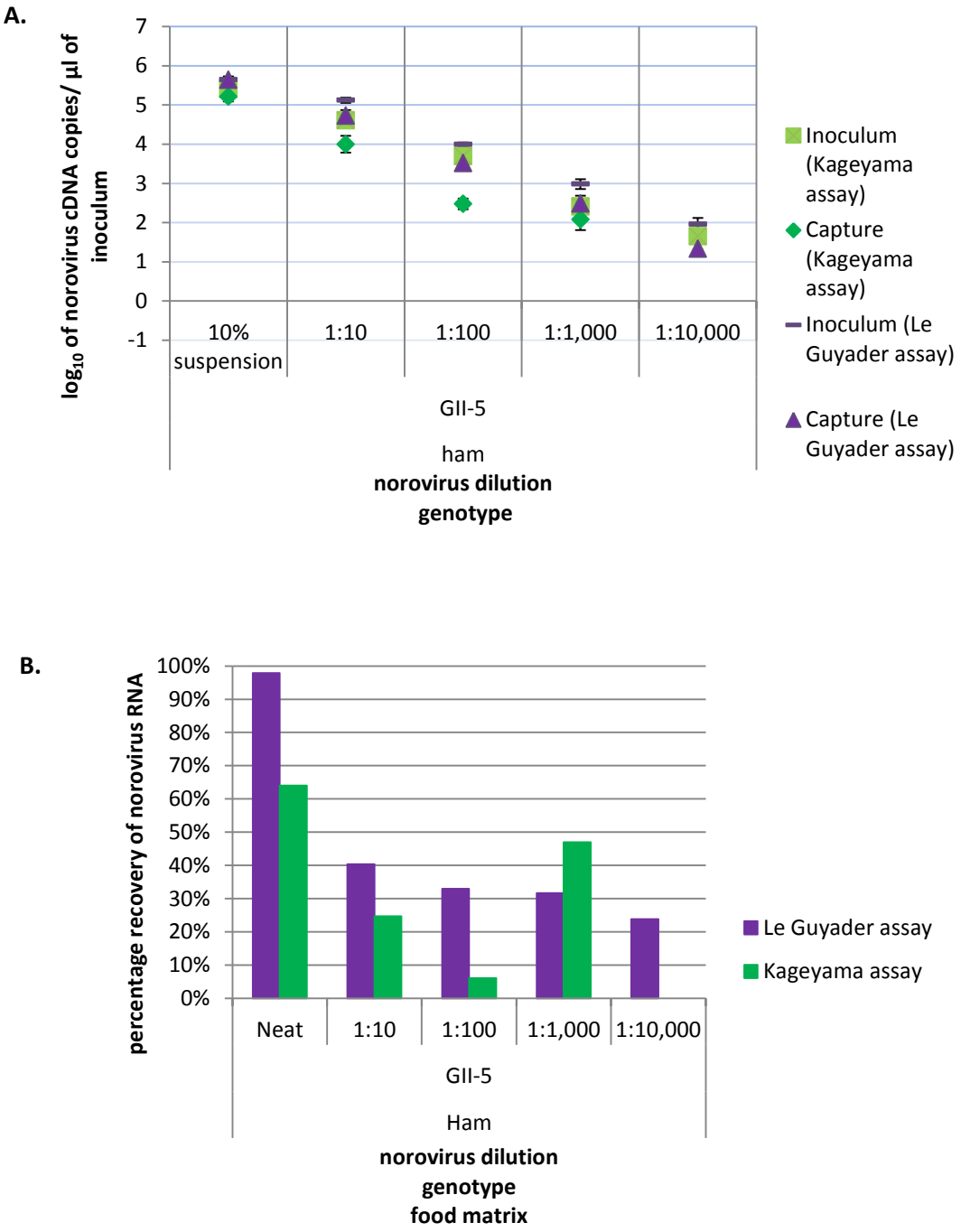
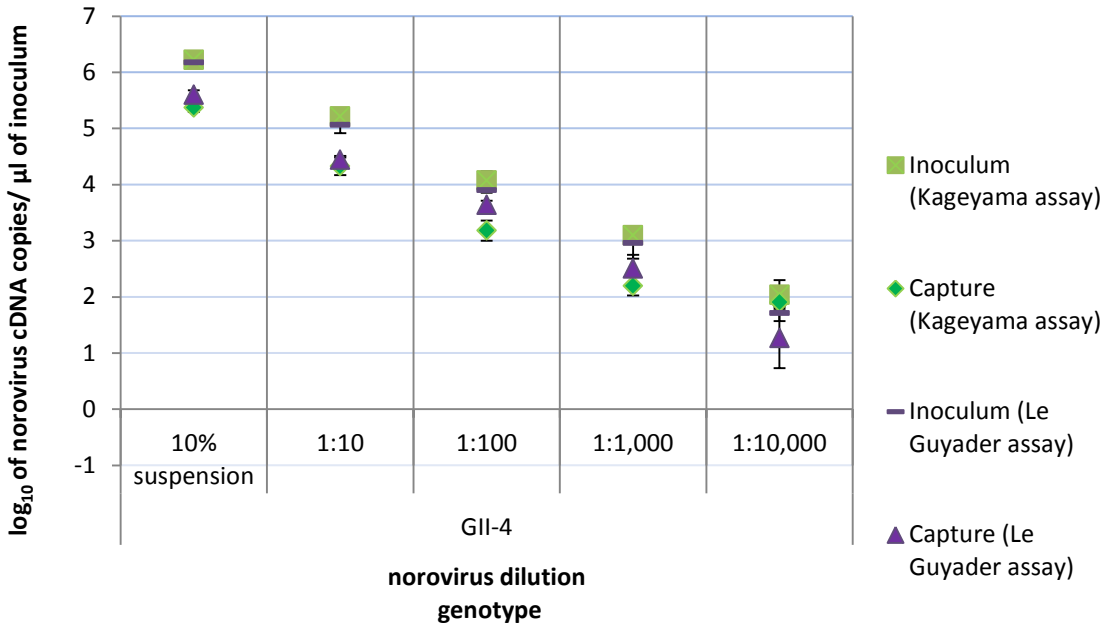


Figure 22 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GII-5 captured from artificially contaminated ham by PCR detection using the Le Guyader or the Kageyama PCR assay. Error bars=SD. Kageyama capture not detected at 1: 10,000 dilution. B. figure representing the percentage recovery on the ham from inoculum using the Le Guyader or the Kageyama PCR assay

A.



B.

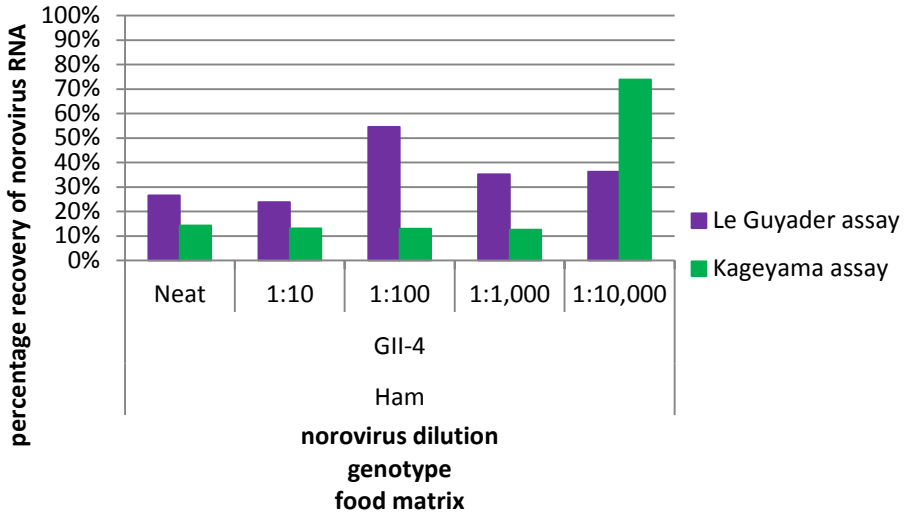


Figure 23 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GII-4 captured from artificially contaminated ham by PCR detection using the Le Guyader or the Kageyama PCR assay. Error bars=SD. B. figure representing the percentage recovery on the ham from inoculum using the Le Guyader or the Kageyama PCR assay

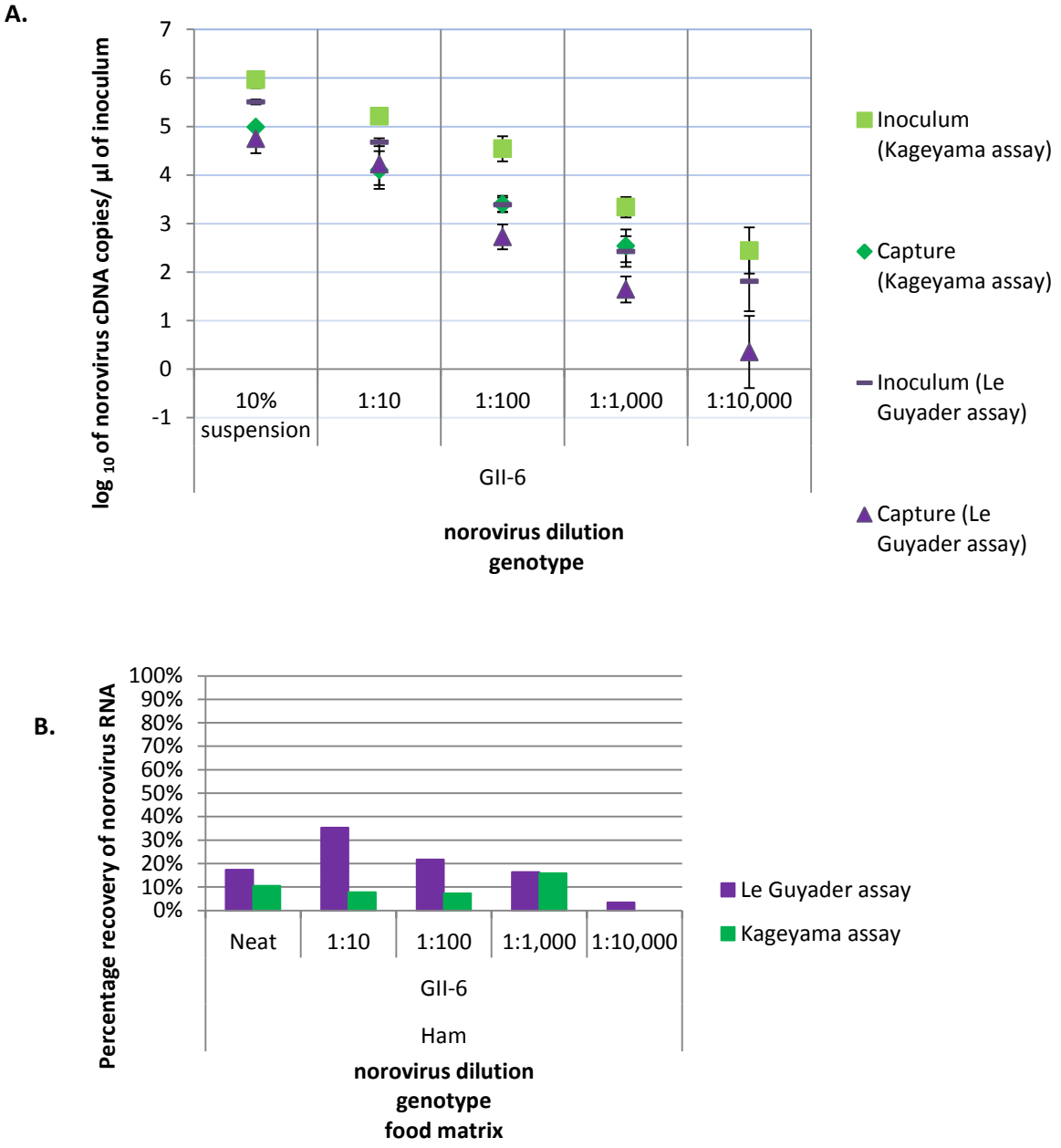


Figure 24 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GII-6 captured from artificially contaminated ham by PCR detection using the Le Guyader or the Kageyama PCR assay. Error bars=SD. Kagyeama capture not detected at 1: 10,000 dilution B. figure representing the percentage recovery on the ham from inoculum using the Le Guyader or the Kageyama PCR assay

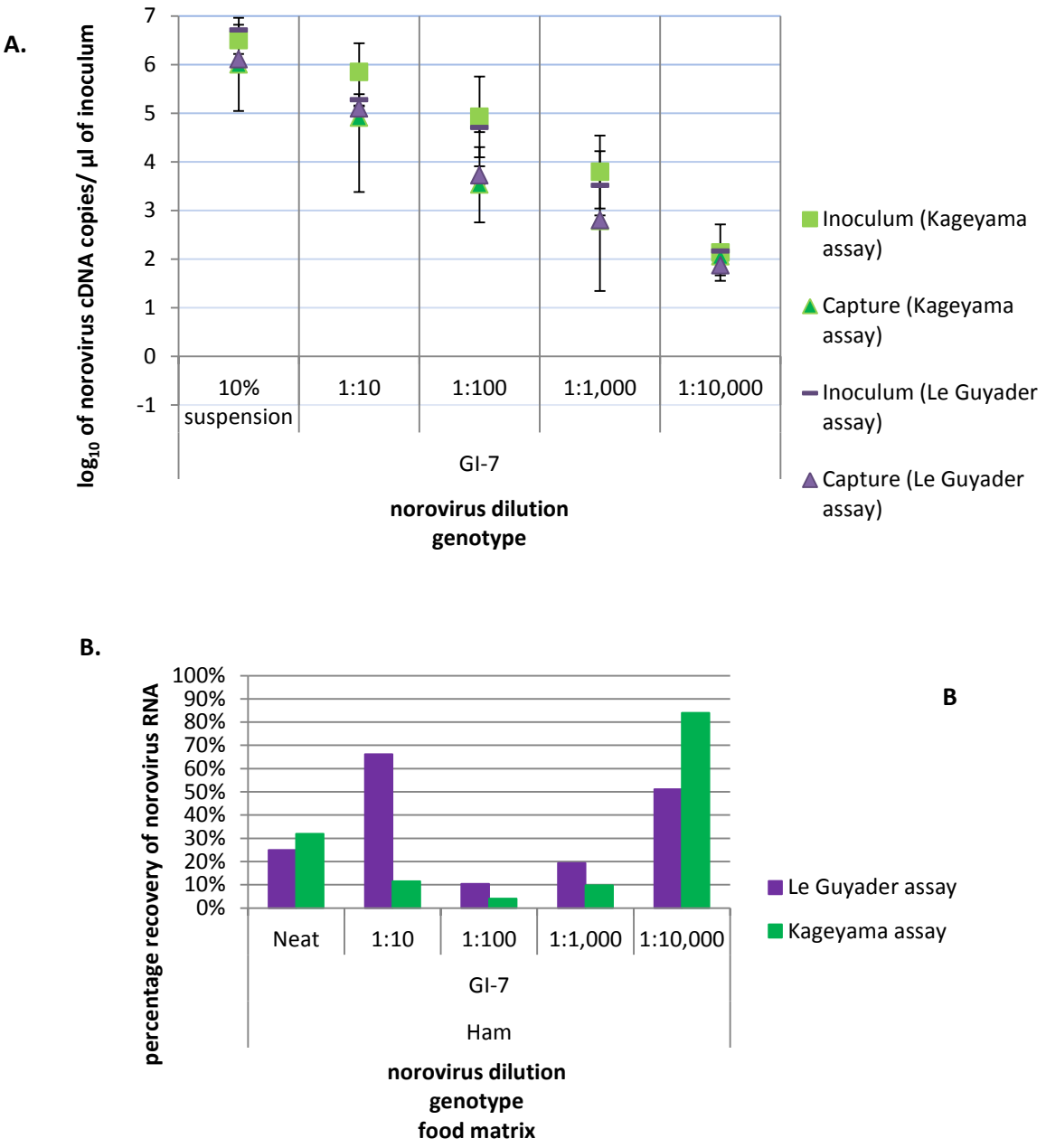


Figure 25 A. figure representing the $\log_{10}$ cDNA copies per µl of inoculum from a ten-fold dilution series of norovirus GI-7 captured from artificially contaminated ham by PCR detection using the Le Guyader or the Kageyama PCR assay. Error bars=SD. B. figure representing the percentage recovery on the ham from inoculum using the Le Guyader or the Kageyama PCR assay.

Table 20 A summary table of the detection limit of norovirus detected in inoculum and by capture from artificially contaminated strawberries, lettuce or ham with norovirus genotypes GII-5, GII-4, GII-6 and GI-7 and the percentage recovered. Dark green indicates the highest percentage of norovirus detected light green indicates the lowest percentage of norovirus detected.

Food type	genotype	average input cDNA copies per µl of inoculum ± SD (Kageyama assay)	Limit of detection of cDNA copies per 25g of food (Kageyama assay)	Percentage recovered (input Cdna copies per µl inoculum/ limit of detection of capture x100)	average input cDNA copies per µl of inoculum ± SD (Le Guyader assay)	Limit of detection of cDNA copies per 25g of food (Le Guyader assay)	Percentage recovered (input Cdna copies per µl inoculum/ limit of detection per 25g of food x100)
Strawberries	GII-5	$3.9 \times 10^2 \pm 2.9 \times 10^2$	$8.5 \times 10^1 \pm 6.2 \times 10^0$	23%	$1.3 \times 103 \pm 5.0 \times 10^1$	$1.6 \times 10^2 \pm 9.0 \times 10^1$	12%
lettuce	GII-5	$3.2 \times 10^2 \pm 2.9 \times 10^2$	$4.3 \times 10^1 \pm 5.3 \times 10^1$	13%	$3.1 \times 10^1 \pm 1.1 \times 10^1$	$2.6 \times 10^1 \pm 9.3 \times 10^{-1}$	84%
ham	GII-4	$1.1 \times 10^2 \pm 3.1 \times 10^1$	$8.0 \times 10^1 \pm 2.5 \times 10^1$	73%	$4.1 \times 10^1 \pm 1.2 \times 10^1$	$1.6 \times 10^1 \pm 6.0 \times 10^0$	40%
ham	GII-5	$2.6 \times 10^2 \pm 1.4 \times 10^2$	$1.2 \times 10^2 \pm 6.7 \times 10^1$	47%	$9.1 \times 10^1 \pm 4.0 \times 10^1$	$2.2 \times 10^1 \pm 3.0 \times 10^1$	24%
ham	GII-6	$2.2 \times 10^3 \pm 9.9 \times 10^2$	$3.5 \times 10^2 \pm 2.6 \times 10^2$	16%	$6.5 \times 10^1 \pm 5.0 \times 10^1$	$2.3 \times 10^0 \pm 9.0 \times 10^1$	4%
ham	GI-7	$1.4 \times 10^2 \pm 3.7 \times 10^1$	$1.1 \times 10^2 \pm 5.7 \times 10^1$	84%	$3.1 \times 103 \pm 5.2 \times 10^1$	$7.4 \times 10^1 \pm 2.9 \times 10^1$	24%

3.6. Application of the optimised capture, concentration, nucleic acid extraction and detection method on a range of ready-to-eat foods

Food samples representing each of the thirteen HPA (2009) RTE food categories (Figure 26) were artificially contaminated with a norovirus GI or GII positive faecal specimen, for the purpose of assessing the final protocol for the detection of norovirus from a range of RTE food matrices. All tests were done in duplicate and the standard deviation was calculated for each food matrices tested. All food matrices were tested prior to artificial contamination to ensure they were not contaminated at the point of purchase (data not shown). The average number of $\log_{10}$ cDNA copies per 200µl of inoculum of norovirus was plotted and the average number of $\log_{10}$ cDNA copies per 200µl of norovirus inoculum recovered from the artificially contaminated RTE food was analysed; norovirus GI (Figure 26 A.) and norovirus GII (Figure 26 B.). Variability in virus recovery between replicates was small for most food matrices excect for the detection of norovirus GI from cream cake. This could have been due to the high lipid content in this particular food matrix, which may have not been sufficiently removed during the nucleic acid extraction stages and may have caused a greater amount of PCR inhibition. Variability in the percentage of norovirus recovered ranged from <1% to 55% for norovirus GI and <1% to 24% for norovirus GII from different RTE foods, despite a standardised detection method was applied. Different food matrices could interfere with the extraction and PCR detection stages in different ways.

Recovery of GI and GII was possible for all the food categories. For those foods contaminated with GI norovirus, the percentage recovery was <10%, and in 5 of these (sponge cake, sliced ham, smoked salmon, olives in oil and dried fruit) recovery was <1%. Among foods contaminated with GII, these ranged from <1% (5 samples) to 20%, but only two samples (salad with dressing and olives in oil) showed a recovery >10%. No correlation of the recovery rates of norovirus genomes could be seen according to food category.

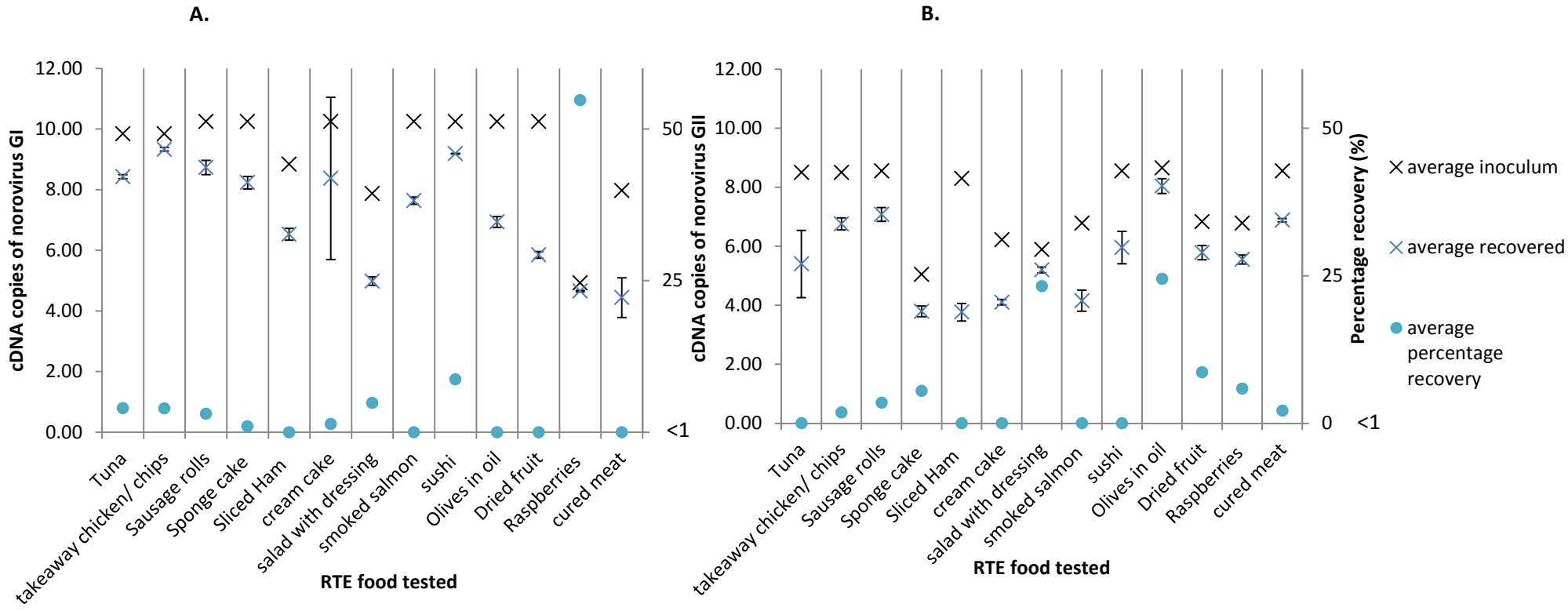


Figure 26 Average $\log_{10}$ cDNA copies of norovirus from two replicates of the input inoculum and the average $\log_{10}$ cDNA copies of norovirus recovered from two replicates of artificially contaminated ready-to-eat foods. A. artificial contamination and recovery of norovirus from RTE food with 200µl of a faecal inoculum containing norovirus GI. B. artificial contamination and recovery of RTE food with 200µl of faecal inoculum containing norovirus GII. Blue circles= average percentage from calculated from two replicates. Error bars= SD

3.7. Discussion

The aim of these studies was to develop a method applicable for the detection of norovirus from a wider range of foods than those currently contemplated in ISO/TS 15216 (2013). In addition, an improved sample throughput was also sought as this is an important consideration for food laboratories, in order to rollout norovirus testing in routine foodborne suspected outbreaks. This was done by considering all the stages of sample processing including the cost, systematically testing and comparing different methods available for each stage: Elution of the virus from the foods, concentration of the virus to a workable volume, nucleic acid extraction and detection by RTqPCR.

3.7.1. Virus elution

Elution of the virus from the food matrix can be difficult as the distribution of virus varies with food type (Bosch *et al.*, 2011, Park *et al.*, 2010, Baert *et al.*, 2008). In most foods contaminated by an external source, virus contamination is uneven and present at low levels compared to those seen within clinical specimens (Glass *et al.*, 2000, Teunis *et al.*, 2008). Considering this, this study evaluated two different food sample preparation methods. The two food processing methods evaluated were homogenisation as used routinely for detection of bacterial and viral contaminants of food, as applied by other authors in some studies (Atmar *et al.,* 1995, Di Pinto *et al.,* 2003, Barnaud *et al.,* 2012); and surface washing as used in ISO/TS 15216 (2013) for the detection of viruses from soft fruit and salad vegetables.

Homogenisation has the advantage of including the entire food item, and this is important in virus that is expected to be internalized or even intracellular, as may be the case for pork

products contaminated with hepatitis E virus. However, considering other enteric viruses such as norovirus, which is the main aim of this work, and hepatitis A virus, these typically human pathogens are most often found contaminating the surface of the foods. Although internalisation of virus contamination via root uptake has been identified in fresh produce, this has only occurred in a small number of samples in irrigation experiments contaminated with very high viral loads (Hirneisen and Kniel, 2013), and in some studies not at all (Chancellor *et al.,* 2006). This suggests that internalisation of virus in fresh produce is rare. In comparison the likelihood of external contamination of fresh produce with faecal material by external sources has been identified in many studies, in particular through contact with sewage or contaminated irrigation water during production. According to El-Senousy *et al.* (2013) green onions, water cress, radish and lettuce irrigated by water from the Nile Delta in Egypt were tested by surface washing of these foods. From this method of virus elution, it was identified that 20.8% to 34.0% of this produce was contaminated with norovirus GI at 10^2 copies per gram of food. Contaminated pesticide is another potential source of external contamination of fresh produce, due to the mixing of pesticide with contaminated water sources such as well, irrigation and river water (Potera, 2013). Although only laboratory studies involving the artificial contamination of pesticide has been conducted in the literature, it has been identified that norovirus GI-4, GII-4 and murine norovirus persisted in artificially contaminated pesticide, and murine norovirus remained infectious in seven out of eight pesticides preparations (Verhaelen *et al.,* 2013).

Another common source of external contamination is through food handling by an infected food handler. Although it is difficult to provide accurate estimates on gastroenteritis caused specifically through an infected food handler, viruses have been implicated in foodborne outbreaks associated with food handler involvement, usually due to bare hand contact and poor hand hygiene (Todd *et al.,* 2007). Virus contamination via an infected food handler

can occur at any stage of production and on any food type, although the risk of contamination increases in foods which are heavily handled, and in those foods which are consumed raw or lightly cooked. Alternatively another source of external contamination can be due to contact with contaminated surfaces and fomites during processing in food preparation environments. Wang *et al.* (2013) identified cross contamination of murine norovirus occurred in up to seven different fresh produce that were successively prepared with a single contaminated knife.

As many sources that may contaminate the external surfaces of foods with faecal material have been identified, all artificial contamination experiments were conducted by spreading the norovirus inoculum across the external surfaces of RTE foods, and showed that norovirus detection was improved by 1-2 logs from three different categories of RTE foods tested by surface washing in comparison to homogenisation (Table 13). This may have been due to an increase in food debris carry over by homogenisation, affecting the efficiency of the virus capture and concentration step, despite attempts to mitigate this by using a filter bag to remove larger particles of food prior to the norovirus capture step. Furthermore, homogenisation may also result in the release of substances and molecules not efficiently removed by the nucleic acid extraction that may inhibit RT-PCR.

Surface washing with 50 ml of sterile PBS was therefore considered fit for purpose and the processing method of choice for the elution of virus from food surfaces. Using smaller volumes for the wash showed poor recovery of norovirus from all foods tested, most likely this volume was not large enough to have thorough contact with all external food surfaces (Figure 10). The efficiency of norovirus detection was not significantly different between 50ml and 100ml wash volumes.

The main limitation of this approach is that it may not be appropriate for food matrices that may be internally contaminated, such as molluscan bivalves. Molluscan bivalves concentrate viruses within their digestive tissues during filter feeding (Torok, 2013). Norovirus contamination of molluscan bivalves during production or harvest by sewage containing water has been identified in many studies (Le Guyader *et al.,* 2006, Escudero *et al.,* 2012, Campos *et al.,* 2016, Boxman *et al.,* 2016). For this reason, the method described in ISO 15216 (2013), in which the digestive diverticulum of molluscan bivalves is dissected and processed, is a more appropriate sample preparation method for this food type. However, there is still potential for molluscan bivalves to become externally contaminated either through contact with other contaminated ingredients, catering surfaces, fomites or through infected food handlers during preparation (Smith *et al.,* 2012). With these contamination sources being less likely to occur this would not justify changing to this method of testing for this particular food group, however the surface wash method is appropriate and applicable to ready to eat foods, for which there is currently a testing gap.

3.7.2. Virus capture and concentration

Surface washing results in a large sample volume in which the virus is likely to be highly diluted, and therefore a concentration method is required. Many capture, and concentration technologies have been described from the literature (Table 1). The method in ISO/TS 15216 (2013) is considered the gold standard and has been applied to bottled water, molluscan bivalves, soft fruits and salad vegetables. This method was trialled with the view to apply it to a wider range of food. It is a non-specific virus concentration method that relies on PEG/NaCl precipitation at high-speed centrifugation. While effective this method has the disadvantage that it is time consuming, the method incorporates the use of chloroform which has health and safety implications, and throughput is severely limited by the need to centrifuge large volumes. Another approach for virus concentration is specific

capture using molecules that have affinity to receptors or epitopes in the virus surface. Such molecules may include antibodies, however, for highly variable viruses such as norovirus this would not be a viable option due to the stringent genotype and variant specificity associated with norovirus-specific antibodies (Allen *et al.,* 2008). Immune-magnetic separation has been used to detect known norovirus genotypes from artificially contaminated food in one study (Park *et al.,* 2008). Although the results of this study found that this method combined with real-time RTqPCR improved recovery rates compared to conventional PCR from 5% for both norovirus GI and GII to 14% for norovirus GI and 30% for norovirus GII, this was only applied to the detection of a single norovirus genotype, one from each genogroup. Furthermore a panel of monoclonal and polyclonal antibodies against various genotypes of norovirus was not validated in this study, but was proposed for future experiments.

In contrast, the interactions between norovirus and HBGAs have been well defined and are broadly reactive (Harrington et al., 2004, Cao et al., 2007). Although strain variability to HBGA binding exists, revalidation of the capture method for new emerging strains is required, as not all noroviruses interact with HBGAs and this is a limitation of the technique (Harrington et al., 2002; Huang et al., 2005). Synthetic HBGA oligosaccharides are not readily available commercially and inconsistencies in assays have been reported (Donaldson et al., 2008, Harrington et al., 2004, Cannon and Vinje, 2008, Lindesmith et al., 2008). To overcome this, PGM was used in this study as a surrogate for synthetic HBGAs. PGM contains a mixture of carbohydrates, including sialic acid, and the different carbohydrates present in this complex mixture may provide binding ligands for other viruses and bacteria.

Based on a protocol described by Tian *et al.,* (2010) that exploits the ability of PGM to bind to human norovirus this method was successfully applied to salad vegetables and sewage

(Tian *et al.,* 2010, Tian *et al.,* 2012). When both ISO 15216 (2013) and Tian *et al.,* (2010) concentration methods were compared head to head, detection of a diluted faecal sample containing norovirus by the Tian *et al.,* (2010) method was non inferior to the ISO 15216 (2013) method (Table 14). Although a norovirus GII positive faecal sample only was used throughout the comparison, this was conducted to maintain sample consistency, which was important when comparing the performance of the two capture methods. Due to health and safety concerns around the use of chloroform as described in the ISO 15216 (2013) method testing was limited. Therefore an extensive comparison of the two capture methods using a panel of faecal samples containing different norovirus strains was not conducted. Bartsch *et al.* (2016) compared five different concentration methods of which both these concentration methods were included in this study, and found that ISO/TS 15216 (2013) resulted in better rate of recovery of norovirus from frozen strawberries at 1.71% (SD ± 2.31) compared to a recovery rate of 0.04% (SD ± 0.10) by the Tian *et al.,* (2010) method. This study only tested three strawberries and a high standard deviation was identified in the recovery of norovirus from these three samples by the ISO/TS 15216 (2013) method in comparison to the Tian *et al.,* (2010) method. The three strawberries were repeatedly freeze-thawed prior to testing; therefore, the integrity of the food was poor prior to molecular detection and may have impacted on the norovirus recovery rates when evaluating both concentration methods. The protocol described in this thesis differs from the published one: here the captured norovirus was directly lysed off the magnetic beads whereas the publish method included 3 washes of the magnetic beads with 1 ml of PBS before the addition of lysis buffer, and this could have resulted in some virus loss.

Capture efficiency at different pH conditions was evaluated. The pH can determine the isoelectric charge and hydrostatic interactions between viruses and glycan conjugates (Vega *et al.*, 2008). Factors such as the food matrix and wash buffer may determine the pH

of the food wash environment. According to Goodridge *et al.* (2004) the isoelectric point of some norovirus GI strains was reported between pH 5.9-6.0 and pH 5.5-6.9 for some GII strains. Of the three pH levels tested, acidic conditions at pH 3.5 were shown to enhance virus capture. Most glycan conjugates recognised by viruses are believed to be negatively charged (Harvey et al., 2011). PGM is believed to be negatively charged at pH 3.5, although the isoelectric point for PGM has been reported between pH 2 and pH 3 (Cao *et al.*, 1999). According to Tian *et al.,* (2010) at pH 3.6 the capsid proteins of both norovirus GI and GII are positively charged. This supports the findings of this study, where an acidic pH improved virus recovery. The use of pH adjustments to aid absorption or elution of the virus on and off solid phases is also exploited in ISO/TS 15216 (2013). However flocculation of virus particles from food surfaces is performed using a tris-glycine wash buffer containing beef extract at pH 9.5, followed by the addition of hydrochloric acid to neutralise the tris-glycine, beef extract buffer to pH 7.2 during virus capture and concentration. Food specimens are processed at this pH with no further pH adjustments to a more acidic environment. In this study, experiments to normalise the pH of a wider range of RTE food surface washes in the presence of the PGM virus capture was conducted to optimise binding efficiency, as it was acknowledged that a wide range of food pH could vary binding efficiency. This thesis wanted to address the capture and concentration of norovirus under optimal binding conditions to improve the recovery of norovirus from large volume food surface washes from a range of RTE foods. It was found that PGM coated beads captured virus more efficiently when the pH food wash buffer was adjusted to pH 3.5, for all matrices tested. This is in agreement with findings by Tian *et al.* (2010) but different to the pH conditions favoured in ISO/TS 15216 (2013). However, the Tian *et al.,* (2010) methods has been validated specifically for testing fresh produce and shellfish, whilst the ISO 15216 (2017) method has been validated specifically for fresh produce,

drinking water, shellfish and swabbed food surfaces. By comparison this study addressed normalising the pH of a wider range of foods to optimise norovirus binding conditions to improve recovery from large volume food surface washes, as the method designed was required for use in testing a wider range of RTE foods as demonstrated in figure 26.

Incubation temperature and time are two additional factors that may influence the interactions between norovirus and the ligand and therefore the capture efficiency. No significant differences were observed when capture was conducted at 4^{o}C or at ambient temperature. Furthermore, as bead capture reagents were stored at 4^{o}C it was decided that all specimens would be incubated at 4^{o}C in order to maintain the cold chain, which is also observed in ISO/TS 15216 (2013). Also, norovirus in faecal or vomit samples are stable from degradation when stored at 4^{o}C for extended periods; therefore it is likely that norovirus stability will be protected in these conditions, which may be particularly important when dealing with samples that contain low viral loads. Tian *et al.,* (2010) conducted capture at room temperature for 15 minutes. In this study, the shortest incubation time was 30 minutes and a slight reduction in capture efficiency was identified when specimens were incubated for this length of time in comparison to 60 minutes, which was determined the optimum incubation time. Capture for longer periods did not provide any benefit, and higher temperatures (37^{o}C), had a negative impact on the capture efficiency (Figure 13).

Bead saturation has been reported using PGM in specimens containing 7 log cDNA copies/ml by Zhou *et al.* (2017). This was believed to be due to an excess of viral particles beyond the binding space available on the HBGA-coated magnetic beads. It may be possible to overcome this by increasing the volume of beads used in capture or diluting the sample by a factor of ten when this is a problem. However, in the context of the detection of norovirus in food surfaces, it is unlikely that such high viral loads will be found, and in

practical terms, the capacity of the method described here should be adequate for the intended purposes. Finally the optimal conditions for virus capture and concentration were established as incubation of 0.15mg/ml of PGM conjugated magnetic beads with 50ml of specimen wash at pH 3.5 and 4^0C for 60 minutes.

Overall, the method described here appears to be at least as good as ISO/TS 15216 (2013) method with no significant virus loss, but reduces processing time significantly by 2 hours and 20 minutes, as well as increasing throughput capacity considerably. High throughput testing is an important consideration when planning to incorporate virus testing from foods to a service laboratory: current throughput capability of each of the Official Control Laboratories for food in England each year is approximately 30,000 specimens.

3.7.3. Nucleic acid extraction

The role of the automated nucleic acid extraction is to remove inhibitors so that they are not carried through to the PCR stage for nucleic acid target specific detection. Fully automated extraction methods combined with real time RT-PCR offer the advantage of consistent sample processing, increasing productivity, reducing human error, and minimising cross contamination compared to processing using manual methods. These benefits have increased the use of fully automated nucleic acid extraction platforms in most diagnostic laboratories. All four platforms evaluated were based on the method first described by Boom et al. (1990); guanidinium isothiocyanate in combination with size fractionated silica. Three of them used magnetic silica, whilst the fourth, the QIAgen QIAxtractor™ utilises silica filtrations technology combined with vacuum aspiration. Few studies have compared the performance of these platforms for the extraction of nucleic acids in food or food derived samples (Marshall and Bruggink, 2006). Initial evaluation for the detection of norovirus from faecal specimens demonstrated inferior performance of two of the platforms (the QIAgen QIAxtractor™ and Promega Maxwell 16™), as these

platforms were unable to detected norovirus ten-fold less than the Roche Mag*NA* Pure MP96™ and Qiagen QIAsymphony™ (Figure 16).

Subsequent evaluation of the sensitivity of the Roche Mag*NA* Pure MP96™ and Qiagen QIAsymphony™ extraction platforms with 288 specimens demonstrated both platforms and associated extraction protocols for faecal samples were equally sensitive. This is also in agreement with previous studies (Verheyen *et al.*, 2012, Witlox *et al.*, 2008). However, when evaluating these extraction platforms for viral nucleic acid extraction from food washes, it became apparent that the Roche Mag*NA* Pure 96™ was inferior to the QIAgen QIAsymphony™. This difference in performance, a 4 $\log_{10}$ loss of sensitivity of the Roche Mag*NA* Pure 96™ platform was associated with the change of extraction kit and protocol from the small volume kit to the large volume kit, used for the extraction of faecal samples and food washes in parallel. The transition to the DNA and Viral RNA large volume kit and Universal Pathogen 500 extraction protocol was required as the processing of 200µl of faecal sample, without the addition of lysis buffer prior to total nucleic acid extraction using the small volume kit was deemed a health and safety risk. Therefore a transition to the large volume kit and Universal Pathogen 500 extraction protocol allowed the addition of 300µl of lysis buffer to the 200µl input volume of faecal samples to overcome this operational issue. Although the capture of norovirus capsids from food washes using PGM magnetic beads allowed flexibility in the volume of lysis buffer that the magnetic beads could be re-suspended in, the minimum input volume required by the Universal Pathogen 500 protocol was 500µl. In summary although it is scientifically possible to use the small volume extraction kit which was comparable to the QIAgen QIAsymphony™ to lyse captured norovirus capsids off of the PGM magnetic beads, this protocol raised health and safety risks when processing faecal specimens in parallel in the absence of lysis buffer. Therefore the large volume kit used in combination with the Pathogen Universal 500

extraction protocol was required to process both analytes safely using the same extraction protocol. The use of the Roche Mag*NA* Pure 96 large volume kit failed to detect norovirus from artificially contaminated raspberries and was believed to be due to the transition to the large volume kit which may have diluted out the contamination of norovirus, however the raspberries could have also interfered with the extraction performance (Figure 17).

3.7.4. PCR detection

PCR detection is the gold standard for norovirus detection, it has exquisite sensitivity to detect low viral loads and currently due to the lack of a routinely applicable cell culture model for norovirus, it is the method of choice in clinical diagnostic laboratories, and also for the detection of virus in foods (Bosch *et al.,* 2008). As previously discussed, the PGM capture, whilst providing efficient norovirus capture, it is not fully norovirus specific and will potentially capture other microorganisms. Therefore the PCR will provide further sensitivity and also specificity. In this thesis, the performances of the Kageyama *et al.,* (2003) and the Le Guyader *et al.,* (2009) PCR assays were determined to be of equivalent sensitivity for the detection of norovirus from faecal specimens based on their sensitivity for the detection of four norovirus genotypes (Figure 19). Although the percentage recovery of norovirus detected by the Kageyama assay were higher in faecal specimens contaminated with norovirus genotypes GII-4, GII-6 and GII-7 compared to the Le Guyader assay, this was only statistically significant in the detection of norovirus GII-6 (one-way ANOVA test; $p=0.005$, $p<0.05$). Faecal specimens are complex matrices; however, the complexity of the food matrices presents new challenges. Sensitivity was lost with both PCR assays when used for the detection of norovirus in artificially contaminated foods, with no particular method associated with greater loss of detection than the other. It is plausible

that the loss in sensitivity could be due to the fact the PCR signal in this protocol is generated only from nucleic acid associated with intact norovirus capsids. Norovirus RNA either free or associated with partially degraded viral particles that would be detected in the nucleic acid extracted from the inoculum used for contamination of the foods, will not bind to the PGM and will be lost in the discarded PBS wash. It is also possible that the loss in sensitivity observed in both assays when applied to food could be due to the assay design which was developed with faecal specimens in mind, which contain much higher viral loads; therefore optimisation of the assays for use on food samples may be required.

Both PCR methods in combination with PGM capture were capable of detecting virus contamination of RTE foods and demonstrated equivalent sensitivity for the detection of norovirus from the three food categories tested. The application of a similar method has only been applied to artificially contaminated oysters in a study by Zhou *et al.* (2017) in which PGM was used to capture norovirus from artificially contaminated shellfish and detected using the Kageyama assay. The results of the percentage of virus recovered were 33.3%, 25.0%, and 19.4% from contaminating GI viral loads of 10^5 Viral genome copies per ml of norovirus (Zhou *et al.,* 2017). The recovery rates achieved in the various artificially contaminated food categories in this thesis was in similar range regardless of the concentration of the inoculum, although the limit of detection was compromised with low load inoculums.

Quantification is important in assessing the sensitivity of a detection method, and this is particularly important in the context of food associated viruses, where low level and uneven contamination may be present. The quantification standards used throughout this thesis were dsDNA plasmids (as defined in Table 3). It is acknowledged that there are limitations to using dsDNA plasmid standards to quantify target norovirus in specimens. As dsDNA standards are subjected to the PCR step only, and do not reflect the genomic

structure of the target organism. However, in a study conducted by Costafreda *et al.* (2006), the use of RNA standards compared to dsDNA standards for the purpose of quantification did not result in significant differences. Alternative standard control materials are available that could also be used once validated on the full protocol. These include LENTICULES^{TM} (Hartnell *et al.*, 2012) or standard controls generated by The National Institute for Biological Standards and Control (NIBSC) (Fryer *et al.,* 2008).

Despite the many advantages of the sensitivity of PCR for the detection of viruses, the limitation of this technique is that it is designed to detect viral nucleic acid and is unable to differentiate between infectious and non-infectious virus. Without a cell culture system, it is not possible to determine the proportion of infectious norovirus captured from foods. Therefore, we can only report that the foods were positive for norovirus RNA, highlighting the risk of infection involved in the consumption of that particular food. No further assessment can be made with any great certainty regarding the infectious dose within contaminated food. Interpretation of what proportion of positive PCR signals are infectious is impossible in the absence of a cell culture model and norovirus PCR signals may be generated from a mixture of infectious virus particles, and interfering defective virus particles (Knight *et al.,* 2013). The bead capture technique in this thesis allows the detection of norovirus RNA associated with intact virus capsids, therefore eliminating the possibility of detecting free RNA or damaged virus particles; although it is not possible to determine what proportion of those intact virus capsids are infectious. Alternatively molecular approaches such as use of photoactivatable dyes; which fluoresce when excited at the appropriate wavelengths to assess the infectivity of norovirus, are being developed and may help to differentiate between PCR signals generated by infectious and non-infectious material. Human enteroids as a cell culture model are also under development (Ettayebi *et al.,* 2016). These could be used in the recovery of viruses from foods and could

be used to reduce over estimation of norovirus recovered from foods by RTqPCR (Randazzo *et al.,* 2016). An assessment of these methods in light of the full protocol could be conducted in future work. Until then questions around infectivity still remain.

3.7.5. Selection of the final protocol for detecting norovirus from various food categories

Novel elements of the work presented here are the processing of RTE foods including meats, oily fish, cakes, dried foods, and the incorporation of an automated extraction method.

Although different categories of RTE foods were tested, it was identified that recovery of norovirus was greatest in high risk RTE foods such as fresh produce and salad vegetables. Methodologies for the detection of norovirus from these food groups have been well described in the literature (Sánchez *et al.,* 2012, Martin-Latil *et al.,* 2012, Croci *et al.,* 2008, Butot *et al.,* 2007), possibly due to the high risk of these food types becoming contaminated from external sources. However this thesis applied the full protocol to the detection of norovirus from a range of RTE food matrices beyond those described in ISO 15216 (2013), as there is epidemiological evidence that other foods are likely to be frequently associated with norovirus outbreaks. Technical limitations and the unavailability of a validated method for foods other than those contemplated in the ISO 15216 method has to date been a barrier for the identification of a food vehicle in many suspected foodborne outbreak investigations. Although some studies have more recently begun to develop detection methods for foodborne viruses from food other than fresh produce and salad vegetables (Saito *et al.,* 2015, Stals *et al.,* 2011), detection methods from a broad range of food is important in order to fully understand the burden of foodborne viruses in food as a transmission vehicle, and the public health implications. Foods that resulted in poor norovirus percentage recovery included canned fish and dried fruit. Poor recovery in

foods such as canned fish could be due to the high salt content of the RTE food; however canned products are considered less at risk of becoming contaminated from external sources such as food handler contamination, in comparison to other RTE food categories such as fresh produce and salad vegetables. Canned products are incorporated in the HPA RTE food guidelines for bacterial pathogens in the event that the canning process fails to prevent bacterial growth. Due to the inability of viruses to grow on food, the most plausible source of external contamination of a canned product could be via an infected food handler. Recovery from GI and GII contamination from dried fruits was also low; Dried fruit contains sulphites, which may have acted as PCR inhibitors not successfully removed during the nucleic acid extraction process. Cream cake and other foods which contained cream based fillings showed poor norovirus recovery; this may be due to the high lipid content interfering with the nucleic acid extraction efficacy. Different percentage recoveries from different RTE foods demonstrate that the application of the process requires validation for each new food type. Recovery of norovirus GI from raspberries was an outlier food type, which resulted in 55% recovery, whilst the percentage recovery of other food types was lower (Figure 26). This could have been due to natural contamination of the fruit, although a 25g subset of all foods were tested in parallel to artificial contamination, and it was identified that the raspberries were norovirus negative prior to artificial contamination. However as highlighted in the challenges of testing food virus contamination can be unevenly distributed, therefore the possibility of natural contamination cannot be eliminated. The minimum requirement to fulfil UKAS accreditation is a method validated on 30 different foods. A fully validated method to detect norovirus from food could then be applied alongside validated methods for the detection of norovirus from clinical specimens to improve outbreak investigations.

Modifications to the preparation of certain foods may be required prior to virus capture, in order to improve the sensitivity of detection methods. Some modifications already exist to improve the detection of bacterial targets in the Food, Water and Environment Laboratory Network. Specifically, the addition of skimmed milk powder to cocoa and chocolate based products is conducted to reduce bactericidal properties for the detection of *Salmonella.* Potassium sulphite is added to onion and garlic based products to reduce bactericidal properties for the detection of *Salmonella.* Modifications for other food groups may be required and validated to improve the sensitivity of norovirus detection by PCR. This may include the use of carrier RNA which may improve extraction sensitivity of PGM captured food washes.

4 Application of the detection method in simulated food handling experiments

4.1 Background

Contamination of food with norovirus can occur throughout production, processing and service. Potential routes of transmission include food handlers, other food ingredients and the environment. It can be difficult to identify a certain food item as the source in a norovirus outbreak as food handler transmission often overlap with other routes of transmission such as person to person. Food handlers can be involved in many different stages throughout food production, and outbreaks have been linked to food handler involvement, most commonly due to bare hand contact combined with poor hand hygiene (Todd *et al.*, 2007). Food associated outbreaks have been implicated, in restaurants and other commercial catering premises for example an outbreak of norovirus that occurred in the Fat Duck restaurant (Smith *et al.,* 2012). Despite there being a duty from food catering operators to notify Local Authorities in a timely manner of a suspected outbreak, it can be difficult for authorities to obtain access to all foods and identify contaminated food items or areas of bad catering practice, which may have been the cause of gastrointestinal disease (Smith *et al.,* 2012). Many questions still remain unanswered around this route of transmission. It remains unknown what proportion of contamination typically moves from food handlers to food, and how much gastroenteritis caused by norovirus is attributable to food contaminated by food handlers. There is evidence of food and fomite contamination in the literature, and levels of contamination can be assessed through the use of quantitative methods, however there is limited information on the viral load transferred by hand contamination during actual food preparation. Quantitative data on norovirus transfer from gloved hands to commonly used catering equipment has been published, through simulated food handling experiments (Rönnqvist *et al.*, 2014, Sharps *et al.*, 2012, Stals *et al.*, 2013). Sharps *et al.* (2012) used a high titre inoculum containing 10^{11} genome

copies per ml of norovirus GII and a lower titre inoculum of 10^8 genome copies per ml of norovirus GI, and identified norovirus transferred to other surfaces at viral loads of 10^4 genomic copies per ml for norovirus GI and 10^5 genomic copies per ml for norovirus GII, indicating a potential food safety risk. Other studies have represented transfer efficiency on food contact surfaces as a percentage of the amount of virus inoculated onto a donor surface. Rönnqvist *et al.* (2014) inoculated donor surfaces such as both right and left latex gloved hands with 3.5 $\log_{10}$ PCR units of norovirus GII-4 and identified different transfer efficiencies to other surfaces: latex gloves 33% (± 10%), plastic 27% (± 8%), stainless steel 62% (± 13%), and cucumber 22% (± 7%). This variation in norovirus transfer efficiencies to different surfaces has also been identified by others. Stals *et al*. (2013) inoculated 20µl of 6.6 x 10^6 genome copies of norovirus GII and also identified variable transfer efficiencies from the following surfaces recovered by the PEG precipitation method: nitrile gloves 38% (± 14%), stainless steel 11% (± 3%), boiled ham 8% (± 2%), sandwich bun 20% (± 18%). Although both studies highlight the transfer of norovirus can vary depending on contact surface and viral load, information in both studies takes into consideration norovirus transfer during food preparation, and could be referenced in infection control documents, such as risk assessments on RTE food preparation. Other authors using viruses grown in cell culture and plaque assays to assess virus recovery and infectivity have been conducted through hand contamination experiments. Rotavirus transfer efficiencies ranged from 1.8% to 16.1% transfer from fingertips to a clean disk (Ansari *et al.*, 1988) and hepatitis A (Bidawid *et al.,* 2000a), transfer efficiency was 9.2% from contaminated fingertips to lettuce.

Other measures of infection control have been put in place to improve hygiene practices. These include the publication 'The General Principles of Food Hygiene' (FAO/ WHO, 2003), a guide for food business operators to reduce the risk of food handler contamination and

the likelihood of virus transmission to others. There are also guidelines to specifically reduce the risk of contamination of food associated viruses; 'The Application of General Principles of Food Hygiene for the Control of Viruses in Food' (FAO, 2008) has been published in the Codex Alimentarius International Food Standards (2012), to emphasise that management strategies for food associated viruses are different to bacterial pathogens. Other infection control measures business operators should follow include the elimination of staff with gastroenteritis from work for at least 48 hours after symptoms have ceased, as food handlers displaying symptoms of gastroenteritis are a high risk for contaminating food and the environment. Although symptoms may have ceased after 48 hours, norovirus shedding can occur for longer periods causing an ongoing public health risk. In a study of individuals who had been experimentally infected, norovirus RNA was present in their faecal specimens 28 days post inoculation detected by RTqPCR (Atmar *et al.*, 2008). Although shedding norovirus RNA may not be an indicator of infectivity, prolonged shedding of norovirus in faecal material increases the risk of norovirus transmission (Rockx *et al.,* 2002, Atmar *et al.,* 2008). Laboratory confirmation indicating norovirus infectivity is not possible until a culture model is developed, until then it will not be known how effective these current guidelines are at reducing risk factors and preventing contamination. In the absence of this information, food business operators can ensure food handler employees receive thorough training on infectivity, transmission and good hand hygiene, by referring to the information available in the guidelines previously described.

Some individuals who have an active norovirus infection can be asymptomatic and therefore will not be readily identified, but will act as a carrier and source of infection. These individuals present a risk of transmitting norovirus to others, and are difficult to identify if they work in food catering environments. This was identified in one study, in which two food handlers at work with gastroenteritis prepared a lunch for up to 450 diners.

These diners were affected with gastroenteritis and it was later identified by retrospective testing of the food handler faecal specimens that they contained a high level of norovirus RNA detected (Chen *et al.*, 2016). Another study identified two food handlers with elevated immunoglobulin A titers to an antigenically related Norwalk-like virus strain were associated with an outbreak of norovirus as they manually prepared the salads. The salads were distributed and gastroenteritis was identified in 333 individuals who consumed them across eight North American states. Only 59 faecal specimens from individuals with gastroenteritis were tested, of which 54% were norovirus RNA positive with a Norwalk-like virus strain (Anderson *et al.,* 2001). Asymptomatic food handlers in catering premises have been identified in other published papers (Jeong *et al.,* 2012, Ozawa *et al.,* 2007). One potential method of eliminating asymptomatic food handlers is through routine testing. In the absence of a cell culture model norovirus is frequently detected through molecular analysis detecting extracted RNA from faecal specimens or environmental swabs. Environmental sampling is a useful tool for identifying norovirus RNA in catering environments, and it can be used as an indicator of the level of hygiene and effectiveness of cleaning practices. Boxman *et al.* (2011) identified from random surveillance of catering establishments in the Netherlands, that 1.5% of environmental swabs were norovirus RNA positive out of a total of 2,496 swabs taken from establishments not previously associated with outbreaks of gastroenteritis. However, virological testing is not currently part of routine hygiene monitoring, there is no guidelines on the detection limits for viruses in food preparation environments, and would be costly. Therefore, it is difficult to identify when virus contamination is a problem and whether hygiene practices are effective at addressing it. Bacterial indicator organism levels are used as a proxy for measuring hygiene, however interpretation of the levels recovered from swabs cannot be interpreted as a proxy for virus contamination (EFSA, 2011).

This chapter investigates the extent of norovirus transmission during food preparation in a mock catering simulation experiment. It assesses the capability to detect norovirus from food handlers, food and the environment during the preparation of RTE food, in the form of a cheese and lettuce sandwich. It also establishes the viral loads recovered on hands, food and the environment by different food handlers at each stage of the sandwich making process. It does this by application of the optimised detection method in chapter 3.

This chapter describes the optimisation and validation of food hand contamination and food simulation experiment design, for monitoring and measuring norovirus transfer. The level of norovirus transfer from contaminated food handlers' hands during food production was identified to determine the viral load transfer to other food handlers, the food matrices and the environment.

4.2 Development of a protocol for identifying norovirus transference by food handler contamination

4.2.1 Food handler contamination: development using UV product

A traceable ultra violet (UV) product applied as a cream was used in preliminary experiments as a proxy for norovirus contamination. It was applied to gloved hands to establish whether transfer would occur, and whether it could be traced throughout the preparation of a sandwich. The transfer of UV cream was visualised under a 4watt UV torch and was shown to transfer from the fingertips of the left gloved hand to the fingertips of the right gloved hand, when hands were pressed together. The UV cream was also transferred onto the lettuce after it had been shredded, the bowl in which the lettuce was placed and the bread during sandwich preparation. The visibility of the UV cream was more difficult on white surfaces such as bread, but it remained visible on both gloved hands after

sandwich preparation. This demonstrated how the movement of a substance on food handler's hands can transfer via food and fomites (Figure 27), particularly as the gloves used throughout simulations were those used in catering facilities. This led to the decision that the concept of the mock catering simulation protocol was suitable for evaluation using norovirus positive faecal specimens.

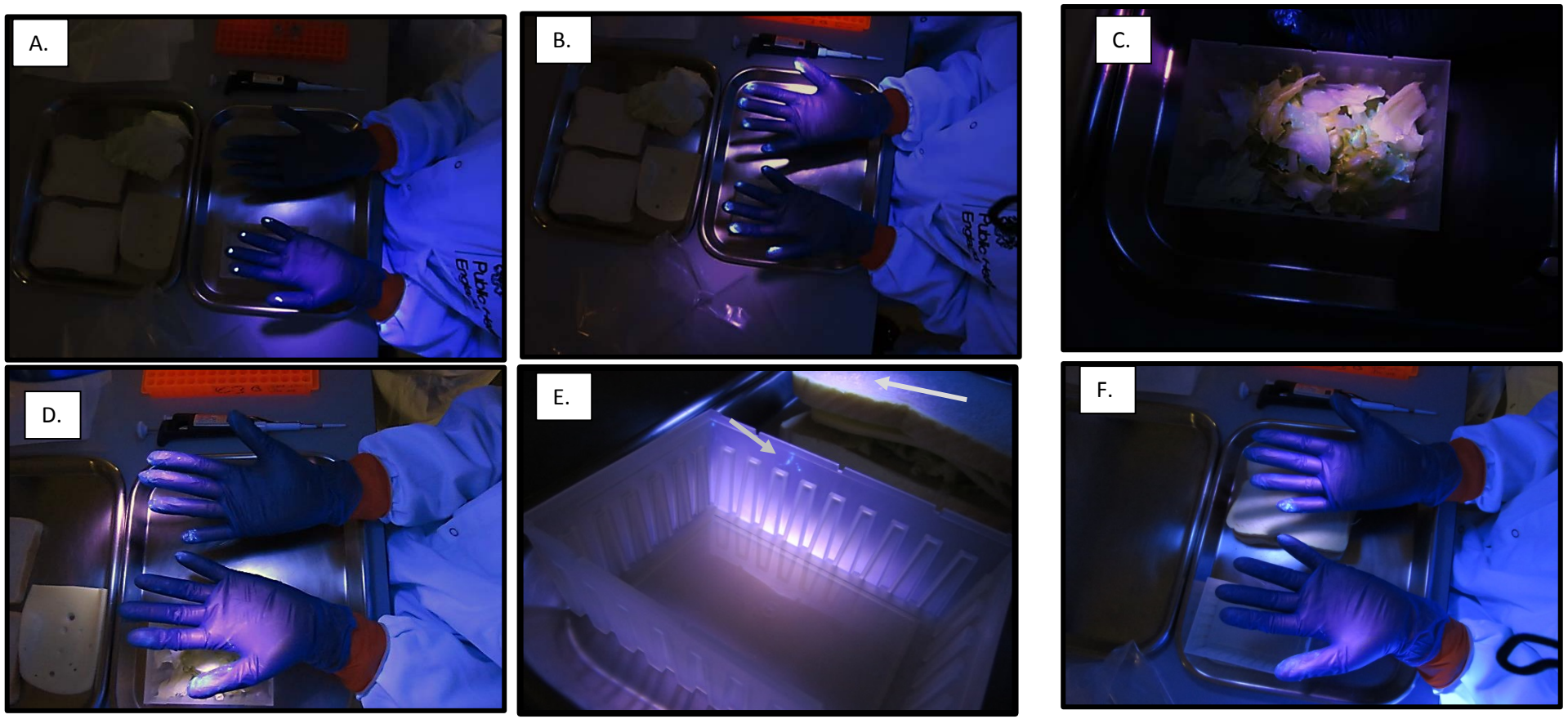


Figure 27 Food handling protocol design images taken under a hand held UV torch to demonstrate transference of UV cream at different stages of the sandwich making process as a visual representation of norovirus transfer. A. The process of inoculating the left gloved hand by pipetting 40µl of UV cream across each fingertip and thumb of left hand. B. It was then transferred to the right hand by pressing the fingertips together for 10 seconds. C. After the lettuce was shredded, transference of the UV cream was evident. D. UV cream still present on both gloved hands. E. The UV cream was also present on the lettuce bowl and bread. The grey arrows show the locations where the UV cream was visualised. F. The UV cream remained on gloved hands after sandwich preparation was completed.

4.2.2 Food handler contamination: validating methods to simulate poor hand hygiene

Experiments were then conducted to identify how to best simulate food handler contamination. This was measured by the average amount of norovirus transferred from the left inoculated hand to the right non-inoculated hand, and the average total amount of norovirus that was recovered from both hands. The Health and Safety Executive (HSE), the UK regulator of health and safety in the workplace, have published commonly missed areas during hand washing (Figure 28) (HSE, 2013). Based on this publication, three commonly missed areas were chosen as inoculation points to test on food handler gloved hands. A total volume of 200µl of norovirus inoculum containing 6.0 (SD ± 0.1) $\log_{10}$ cDNA copies was contaminated onto the left gloved hand only, but the inoculum was administered in three ways, in the form of:

- A 40µl spot onto the tip of each digit (fingers and thumb) of the left gloved hand
- one 200µl spot in the palm of the left gloved hand
- Twenty spots of 10µl across the length of each digit of the left gloved hand

Once the left gloved hand was inoculated by either one of the three ways described above, both gloved hands were pressed together to transfer norovirus onto the right non-inoculated hand. Each contamination method was carried out in duplicate by one food handler conducting all three tasks. The total amount of norovirus retained on both hands was calculated (Figure 29), and the percentage retained on both hands (Table 21). A 40µl spot was pipetted onto the tip of each digit of the left inoculated hand and an average of 5.2 (SD ± 0.63) $\log_{10}$ cDNA copies of norovirus was retained on both hands by this method of contamination. This resulted in a 14% recovery of the inoculum. When an inoculum of one 200µl spot in the palm of the left was administered, an average of 5.1 (SD ± 0.65) $\log_{10}$ cDNA copies of norovirus was retained on both hands was. This resulted in 12% recovery of

the inoculum. In comparison, twenty spots of 10µl of inoculum resulted in an average of 4.8 (SD ± 0.65) $\log_{10}$ cDNA copies retained on both hands, and a 6% recovery of the inoculum. Twenty spots of 10µl inoculated along fingers required a higher level of pipetting accuracy, and there were concerns that the increase in pipetting could lead to operator error. Although pipetting 200µl of inoculum into the palm of the hand was considered to have the minimum pipetting error compared to the other methods, care had to be taken during the hand press to ensure none of the inoculum was lost through run off, when pressing the palms together. In all three hand contamination methods the inoculated left hand contained more norovirus than the amount of norovirus that was transferred to the right hand by the pressing of hands together. These results led to the decision that 40µl per digit (fingertip and thumb) would be used in all future simulation experiments as this method resulted in the most virus retained on both hands and the most norovirus recovered at 14% of the inoculum.

Figure 28 According to research by the UK Health and Safety Executive (HSE), the shaded dark orange areas highlight the most commonly areas missed during the process of handwashing (HSE 2015).

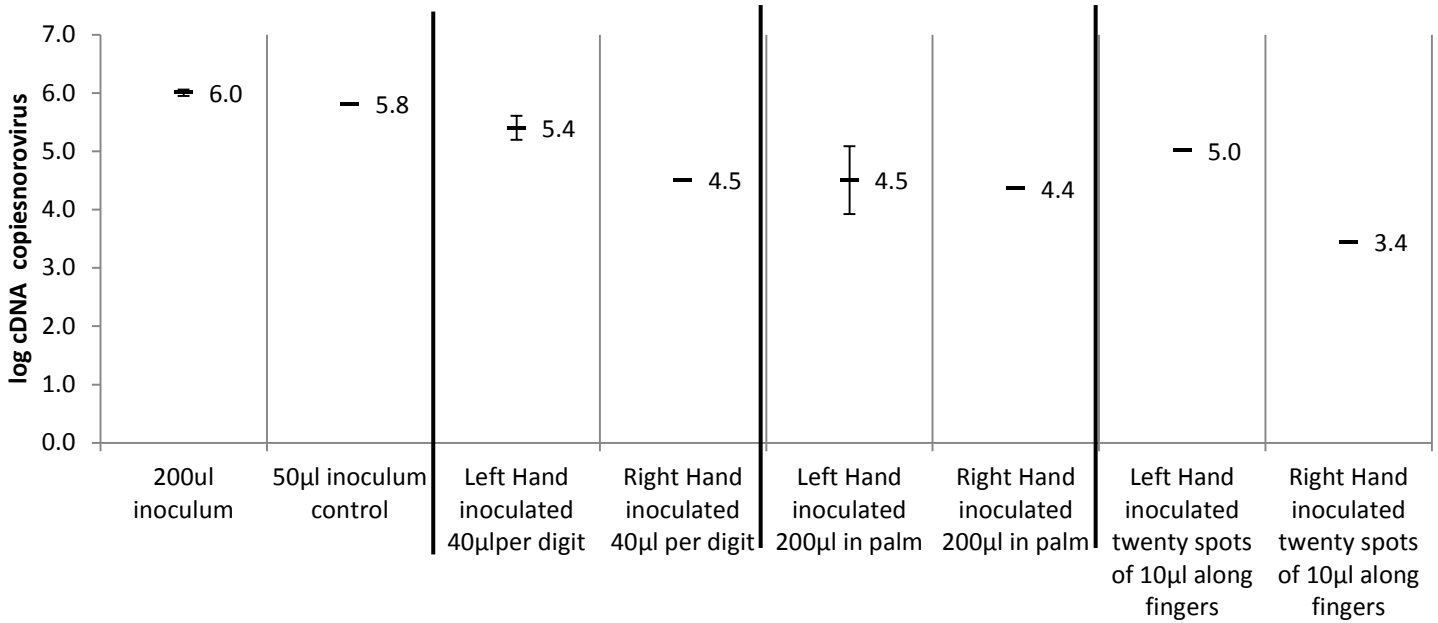


Figure 29 presenting the average amount of norovirus GII retained on both hands after transfer onto both hands from two replicate experiments. The faecal material was administered onto the left glove hand in three different ways, one 200µl in palm, 40µl per digit and twenty 10µl along the length of fingers and transferred to the right hand by pressing glove hands together for 20 seconds, from a norovirus GII containing inoculum. Error bars = standard deviation (SD) (Appendix Q)

Table 21 showing the average log_{10} cDNA copies/ input inoculum, the average viral loads of norovirus GII retained on both gloved hands, and the percentage of virus retained on the hands after artificial contamination of the left hand by three different contamination methods: 40µl per digit; 200µl in the palm; twenty spots of 10µl along the length of the fingers.

Sample	**Average amount of virus in input inoculum** (log_{10} cDNA copies/input inoculum)	**Average amount of virus retained on both hands** (log_{10} cDNA copies/ both hands)	**% retained on both hands** [(total viral load on both hands/input inoculum) x 100]
40µl per fingertip and thumb	6.0 (SD ±0.05)	5.2 (SD±0.63)	14% [(1.42 x 10^5/1.02 x 10^6) x 100]
single 200µl in the palm of the hand	6.0 (SD ±0.05)	5.1 (SD± 0.65)	12% [(1.27 x 10^5/1.02 x 10^6) x 100]
Twenty 10µl spots along the length of the fingers	6.0 (SD ±0.05)	4.8 (SD±0.65)	6% [(5.74 x 10^4/1.02 x 10^6)x 100]

4.2.3 Food handler contamination: identifying norovirus transfer by real time RT-PCR

The protocol trialled in section 4.2.1 of preparing a sandwich was validated using a norovirus GII positive faecal specimen, to act as a proxy for a natural human to food transmission event *in vitro*. This evaluation was conducted with the aim to identify how much norovirus was recovered at each stage of sandwich making using real-time RTqPCR to identify norovirus RNA transfer. Throughout this evaluation, tasks were completed by the same food handler to exclude variation in food handler behaviour. The experiment was set up as shown in section 2.9 dividing the sandwich making into three tasks and completed in duplicate as described below:

- Experimental Stage A = Task 1 only. After inoculation of the food handler's left gloved hand and transfer by pressing both hands together, the food handler shredded lettuce. Once completed, the lettuce and outer, vinyl gloves were removed for testing.
- Experimental Stage B = Task 1 and Task 2. The food handler returned to Task 1 as previously described however this time the lettuce was not removed. At the start of Task 2 prior to preparing the sandwich, the food handler put on a new pair of outer, vinyl gloves, with no additional faecal material being added, to represent a person without soiled hands prior to sandwich preparation. The sandwich was prepared by placing lettuce and cheese between two slices of bread. The gloves and the prepared sandwich were then removed for testing.
- Experimental Stage C = Task 1, Task 2 and Task 3. After completion of Task 1 and 2 as described above, the food handler put on a new pair of outer, vinyl gloves with no additional faecal material being added. The sandwich prepared in Task 2 was

halved and placed into two separate bags for testing. The gloves were also removed for testing.

The experiment was conducted in duplicate and the samples were tested using the methodology developed in Chapter 3. An average viral load from the two replicates for each gloved hand or food item was calculated (Figure 30). The average amount of norovirus from two replicates of the faecal inoculum which was used in the hand contamination experiments contained 6.2 (SD ± 0.01) $\log_{10}$ cDNA copies of norovirus/ µl of inoculum. The percentage of inoculum recovered from both hands decreased after each task from 11.6% after Task 1, 2.8% after Task 2 and 0.02% after Task 3 (Table 22), which was expected due to the increase in handling and contact with different surfaces during the experimental stages of the process. The percentage of norovirus inoculum recovered from the shredded lettuce after Task 1 was 1.9%, however this decreased to 0.1% once the sandwich was prepared (after Task 2) and when the sandwich was halved (after Task 3). Detection of norovirus RNA on the food and both hands in this evaluation demonstrated that the protocol was a valid method for identifying norovirus RNA transfer during preparation of a sandwich.

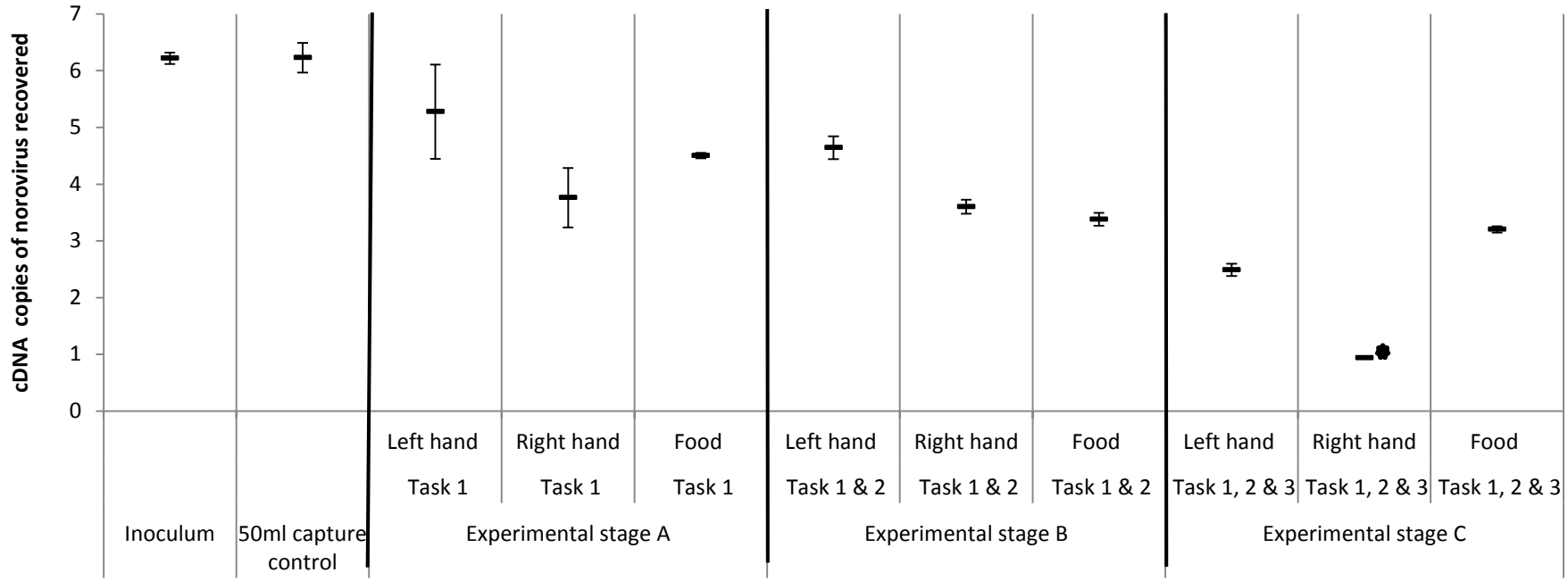


Figure 30 presenting the average $\log_{10}$ cDNA copies of inoculum of norovirus GII transferred during the sandwich making process, which was separated into three tasks and conducted twice by the same food handler completing all three tasks. Error bars = standard deviation (SD); Black arrows indicate the flow of the experiment up to the point where gloves and food were removed for testing.

* = one replicate not an average as norovirus was detected in only one replicate Task 1 food = lettuce, Task 1 & 2 food = whole sandwich, Task 1, 2 & 3 food = sandwich divided in two.

Table 22 presenting the log_{10} cDNA copies of norovirus GII input inoculum recovered on the left hand, right hand and food during sandwich making, segregated into three tasks using a single food handler from two replicates, the average log_{10} cDNA copies of the two replicates, the standard deviation (SD) of the two replicates and the total viral load from gloved hands and food after each task.

	Left hand (log_{10} cDNA copies/hand)				**Right hand** (log_{10} cDNA copies/ hand)				**% recovered on both hands** [(average total amount of norovirus retained on both hands after task(s)/inoculum) *100]	**Food** (log_{10} cDNA copies/ food)				**% recovered on food** [(average total amount of norovirus on food after task(s)/inoculum) *100]
Task number	**Rep 1**	**Rep 2**	**Average**	**SD**	**Rep 1**	**Rep 2**	**Average**	**SD**		**Rep 1**	**Rep 2**	**Average**	**SD**	
Task 1	5.6	4.4	5.3	0.8	4	3.3	3.8	0.5	11.60% ($1.96 \times 10^5 / 1.69 \times 10^6$) x 100	4.5	4.5	4.5	0.0	1.90% ($3.2 \times 10^4 / 1.69 \times 10^{6)}$ x 100
Task 1 & 2	4.8	4.5	4.6	0.2	3.7	3.5	3.6	0.1	2.80% ($4.80 \times 10^4 / 1.69 \times 10^{6) \times}$ 100	3.3	3.5	3.4	0.1	0.10% ($2.4 \times 10^3 / 1.69 \times 10^{6)}$ x 100
Task 1, 2 & 3	4.7	2.6	2.5	1.5	0.9	nvd[1]	n/a[2]	n/a	0.02% ($3.10^2 / 1.69 \times 10^{6)} \times$ 100	3.2	3.3	3.2	0.1	0.10% ($1.6 \times 10^3 / 1.69 \times 10^{6)}$ x 100

[1] nvd=no virus detected, [2]n/a not applicable, Rep = replicate

4.3. Application of the optimised protocol to identify the and quantify norovirus transfer

The optimised protocol was conducted with the aim to identify the level of norovirus transfer from food handler hands, food and the environment by different volunteers. The set-up of the experiment was based on that shown in Figure 6 (Section 2.9) with a modification that involved the use of a different food handler to conduct each of the three tasks of sandwich preparation. The simulation experiments were replicated six times for each norovirus genotype and six times with the molecular grade water in a blinded study. Mengovirus was the molecular internal process control and was detected in all norovirus GI and norovirus GII specimens (Appendix U). Viral loads of norovirus recovered from food handler hands, food and environment were calculated in $\log_{10}$ cDNA copies.

4.3.1. Detection of norovirus GI and GII on hands in simulation experiments

The average amount of norovirus GI $\log_{10}$ cDNA copies from two replicates of the faecal inoculum used in the hand contamination experiments contained 7.8 $\log_{10}$ cDNA copies of norovirus/ µl of inoculum. The percentage of norovirus inoculum recovered from both hands after Task 1 was 9.6% (Table 23). Despite the fact this was derived from the food handling behaviour of six different food handlers, this was similar to the percentage recovered in the evaluation of the protocol in section 4.2.3. The percentage of norovirus inoculum recovered from both hands after Task 2 was 0.02% and 0.004% after Task 3. This was much lower than the percentage recovered in the evaluation of the protocol potentially due to the variation in food handling behaviour by six different food handlers at each task.

The experiment was repeated for norovirus GII using the faecal inoculum used in the hand contamination experiments, which contained an average of 5.9 $\log_{10}$ cDNA copies per µl of

inoculum from two replicates. The percentage of norovirus recovered from both hands from the inoculum after Task 1 was 3.0% (Table 24). The percentage of norovirus inoculum recovered from both hands after Task 2 was 0.06% and after Task 3 was 0.012%. In comparison, the percentage recovery of norovirus GII was less than the percentage recovery observed in the GI simulations and in the evaluation of the protocol experiment. The input inoculum used to artificially contaminate the left hand of Food Handler 1 was two logs lower than that used in the GI simulation experiments (Figure 31 and Figure 32).

The average amount of norovirus distributed on either left or right hand after each task was tested to identify if there was any statistical significance in the amount of norovirus distributed on either hand across the six simulations. An F-test was conducted to see if the variance between the two groups was equal. For the norovirus GI simulations the variance were 3.0 $\log_{10}$ cDNA copies for the left hand and 3.0 $\log_{10}$ cDNA copies for the right hand (F value=1.0 and F critical value=2.3). The F value was less than the F Critical value, therefore the null hypothesis that the variance is equal between the two means is accepted (Appendix W). For the norovirus GII simulations the variance was 1.2 $\log_{10}$ cDNA copies for the left hand and 1.6 $\log_{10}$ cDNA copies for the right hand (F value=0.8 and F critical value=0.4). The F value was greater than the F Critical value, therefore the null hypothesis that the variance is equal between the two means is rejected. This was followed by a two tailed T-test to identify that the difference in means between the left and right hand was not statistically significant for either GI simulations ($p=0.62$, $p>0.05$) or GII simulations ($p=0.08$, $p>0.05$) (Appendix W). These simulation experiments help to interpret the risk associated with norovirus recovery from hands during food preparation, considering the variation in individual's food handling behavior.

Table 23 showing the log_{10} cDNA copies/ hand and the average viral loads of norovirus GI recovered from the left hand and right hand of food handler 1, 2 and 3 from six replicates, and the percentage recovered from both hands

	Average viral load of six replicates			
Sample	**Left hand** (log_{10} cDNA copies/hand)	**Right hand** (log_{10} cDNA copies/hand)	**Average Total amount of norovirus on both hands after task/s** (log_{10} cDNA copies/ both hands)	**% Recovered on both hands** [(average total amount of norovirus from both hands after task(s)/inoculum)*100]
Food Handler 1 (Task 1)	6.6 (inoculated hand)	7 (norovirus transferred)	6.8 (SD ± 7.1)	9.60% ($6.7 \times 10^6 / 6.9 \times 10^7$) x 100
Food Handler 2 (Task 2)	4.4 (norovirus transferred)	3.8 (norovirus transferred)	4.2 (SD ±4.5)	0.02% ($1.6 \times 10^4 / 6.9 \times 10^7$) x 100
Food Handler 3 (Task 3)	3.4* (norovirus transferred)	3.4* (norovirus transferred)	3.4 (SD ± 3.6	0.004% ($2.6 \times 10^3 / 6.9 \times 10^7$) x 100
Average inoculum (log cDNA copies/inoculum)	7.8 (SD ± 0.11)			

*=average viral load calculated from five replicates as no virus was detected on the hands of food handlers in a simulation

Table 24 showing the log_{10} cDNA copies/ hand and the average viral loads of norovirus GII recovered from the left hand and right hand of food handler 1, 2 and 3 from six replicates, and the percentage recovered from both hands

	Average viral load of six replicates		**Average Total amount of norovirus on both hands after task/s** (log_{10} cDNA copies/ both hands)	**% recovered on both hands** [(average total amount of norovirus from both hands after task(s)/inoculum)*100]
Sample	**Left hand** (log_{10} cDNA copies/hand)	**Right hand** (log_{10} cDNA copies/hand)		
Food Handler 1 (Task 1)	4.4 (inoculated hand)	4.3 (norovirus transferred)	4.4 (SD ± 4.7)	3.00% (2.4×10^4/ 8.0×10^5) x 100
Food Handler 2 (Task 2)	2.2** (norovirus transferred)	2.8* (norovirus transferred)	2.7 (SD ± 2.9)	0.06% (4.5×10^2/8.0×10^5) x 100
Food Handler 3 (Task 3)	2.3*** (norovirus transferred)	1.9* (norovirus transferred)	2.1 (SD ± 2.0)	0.012% 1.00×10^2/8.0×10^5) x 100
Average inoculum (log_{10} cDNA copies/inoculum)	5.9 (SD ± 0.11)			

*=average calculated from five replicates no virus was detected on the hands of some food handlers. **=average viral load calculated from four replicates no virus was detected on the hands of some food handlers. ***=average viral load calculated from three replicates no virus was detected on the hands of some food handlers.

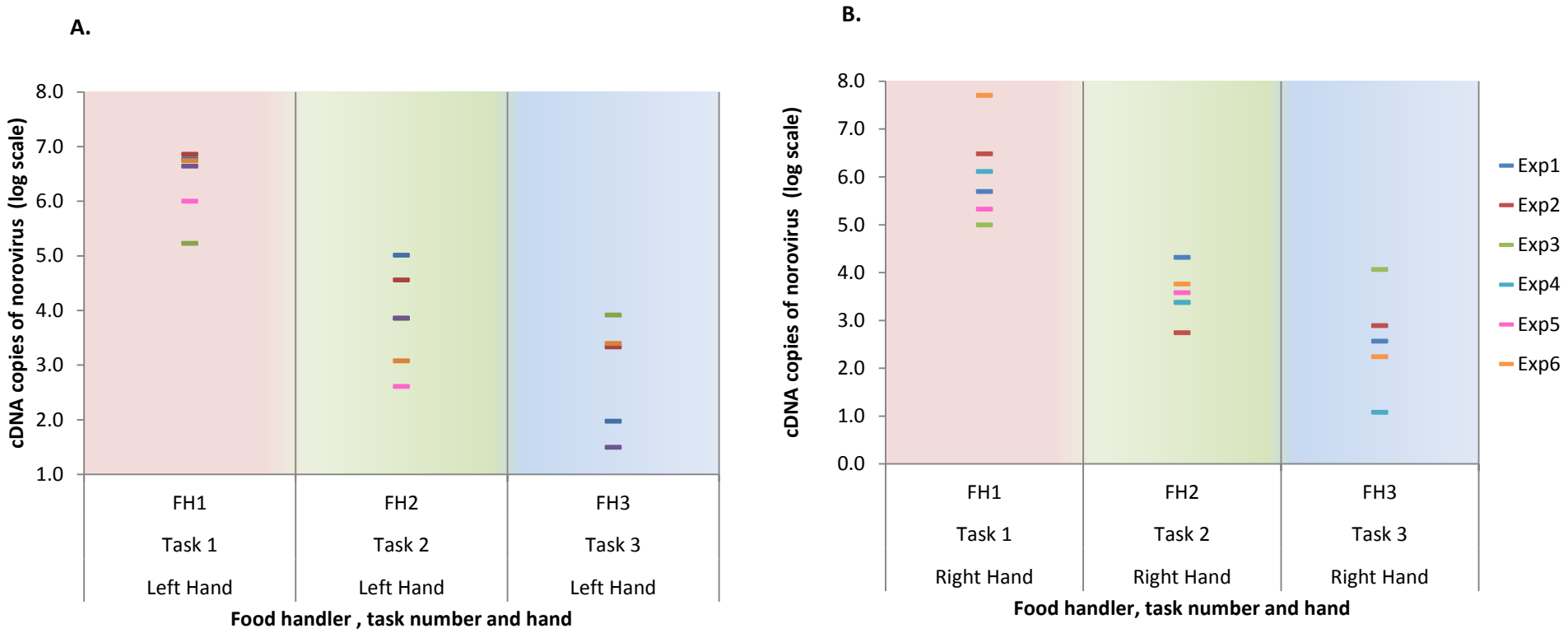


Figure 31 presenting the average viral load in $\log_{10}$ cDNA copies/ and of norovirus GI from six replicate simulation experiments. A. the viral load of each of the six replicates transferred from the left gloved hand. B. the viral load from each of the six replicate transferred from the right gloved hand to demonstrate the amount of norovirus transferred during sandwich making by three different food handlers. Pink = food handler 1 after Task 1. Green = food handler 2 after Task 2. Blue = food handler 3 after Task 3. Crosses represent the average viral load transferred across six replicates of each task (Appendix R).

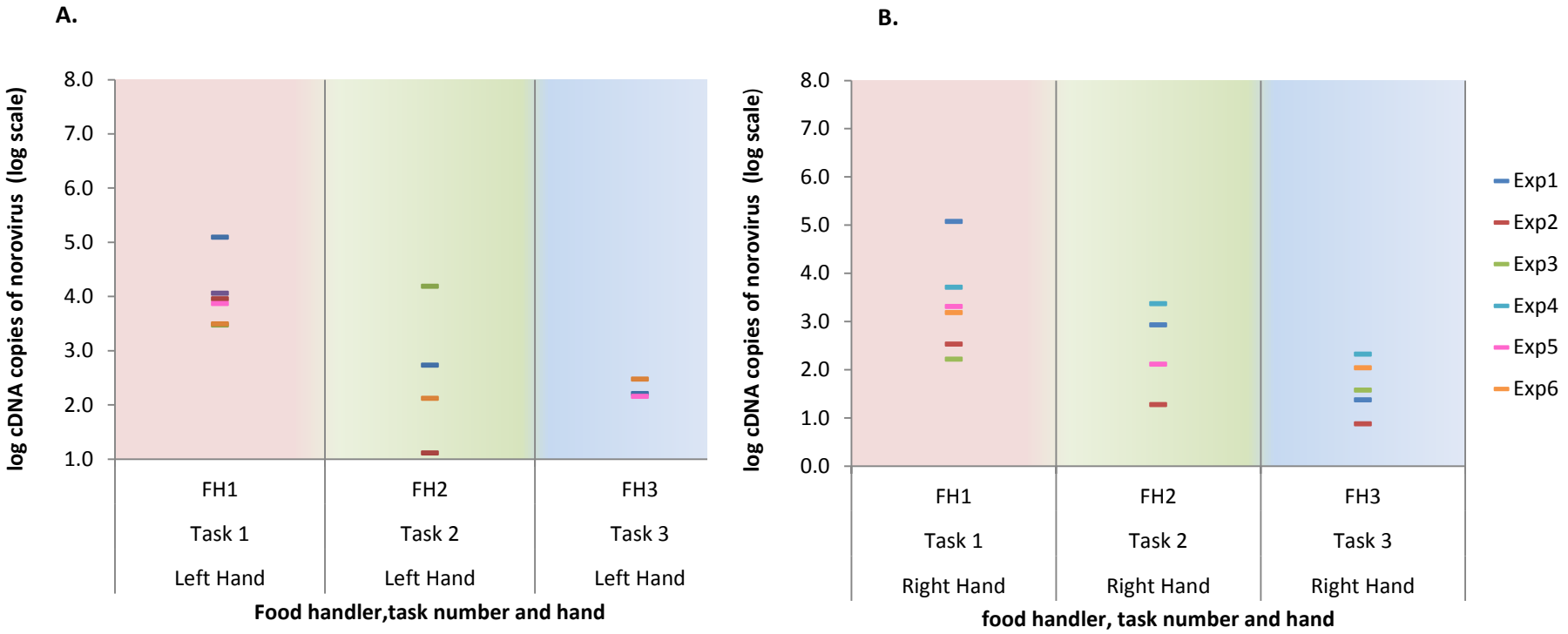
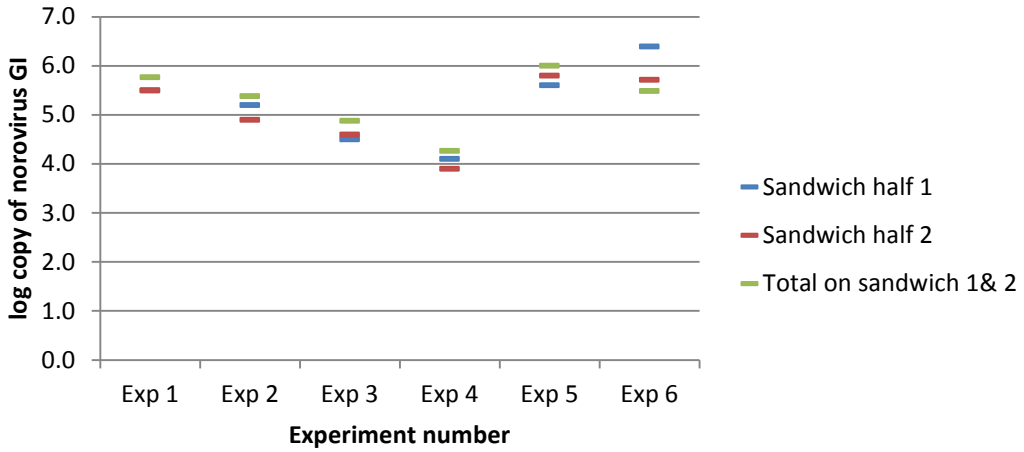


Figure 32 presenting the average viral load in $\log_{10}$ cDNA copies/ hand of faeces of norovirus GII inoculum from six replicate simulation experiments. A. shows the viral load of each of the six replicates transferred from the left gloved hand. B. shows the viral load from each of the six replicates transferred from the right gloved hand to demonstrate the amount of norovirus transferred during sandwich making by three different food handlers. Pink = food handler 1 after Task 1. Green = food handler 2 after Task 2. Blue = food handler 3 after Task 3. Crosses represent the average viral load transferred across six replicates of each task (Appendix S).

4.3.2. Detection of norovirus GI and GII on foods in simulation experiments

Sandwiches that were prepared by the food handlers were divided into two for practical reasons so that they could fit into the stomacher bag for testing, as described in Chapter 3. However, this was also done to represent how sandwiches are normally presented prior to consumption. The amount of norovirus transferred to the sandwich was quantified by how much norovirus was detected from each sandwich piece for norovirus GI and norovirus GII (Figure 33). The total $\log_{10}$ cDNA copies per sandwich 1 and 2 were calculated from the six replicates of the halved sandwiches. From this the average percentage of norovirus inoculum recovered from sandwiches 1 and 2 was 1.2% in norovirus GI simulations (Table 25) and 0.2% in norovirus GII simulations (Table 26).

A.



B.

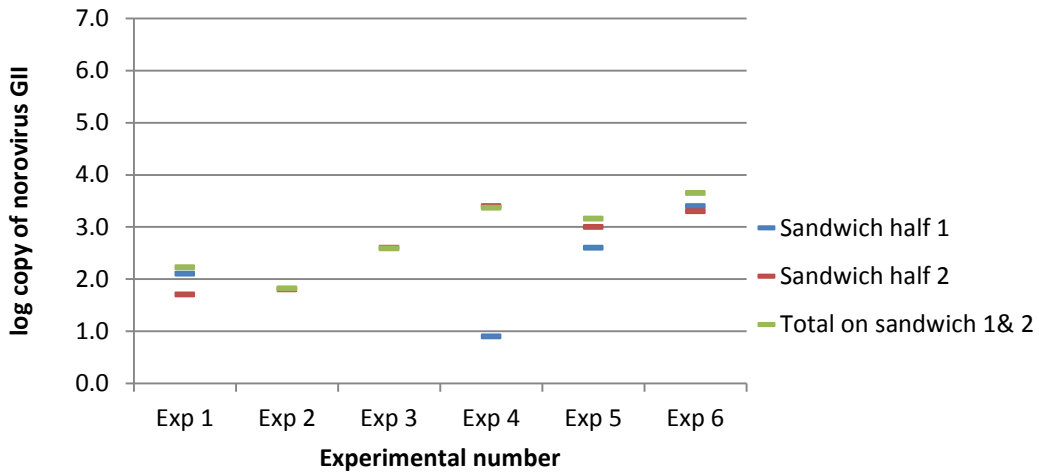


Figure 33 graphs representing the amount of norovirus $\log_{10}$ cDNA copies/ sandwich prepared in the six replicates food handling simulation experiments and total $\log_{10}$ cDNA copies/ sandwich 1 & 2 for A. norovirus GI simulations B. norovirus GII simulations (Appendix T). Exp=Experiment

Table 25 presenting the $\log_{10}$ cDNA copies of norovirus GI detected from each sandwich piece and the total viral load detected from both sandwiches prepared by food handlers in the six GI simulation experiments. Exp = experiment

Norovirus GI	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 4**	**Exp 5**	**Exp 6**
Sandwich piece 1 ($\log_{10}$ cDNA copies/sandwich)	5.5	5.2	4.5	4.1	5.6	6.4
Sandwich piece 2 ($\log_{10}$ cDNA copies/sandwich)	5.5	4.9	4.6	3.9	5.8	5.7
Total on sandwich 1 & 2 ($\log_{10}$ cDNA copies/sandwich 1 &2)	5.8	5.4	4.9	4.3	6.0	6.5
Average on a sandwich piece ($\log_{10}$ cDNA copies/sandwich)	5.9 (SD ± 0.79)					
% recovered on both sandwiches [(average total amount of norovirus from both sandwich halves after task(s)/inoculum) *100]	1.20% $(4.92 \times 10^6/6.04 \times 10^7) \times 100$					

Table 26 presenting the $\log_{10}$ cDNA copies of norovirus GII detected from each sandwich piece and the total viral load detected from both sandwiches prepared by food handlers in the six GII simulation experiments

Norovirus GII	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 4**	**Exp 5**	**Exp 6**
Sandwich piece 1 ($\log_{10}$ cDNA copies/sandwich)	2.1	nvd[1]	nvd	0.9	2.6	3.4
Sandwich piece 2 ($\log_{10}$ cDNA copies/sandwich)	1.7	1.8	2.6	3.4	3.0	3.3
Total on sandwich 1 & 2 ($\log_{10}$ cDNA copies/sandwich)	2.2	1.8	2.6	3.4	3.2	3.7
Average on a sandwich piece ($\log_{10}$ cDNA copies/sandwich)	3.2 (SD ± 0.76)					
% recovered on both sandwiches [(average total amount of norovirus from both sandwich halves after task(s)/inoculum) *100]	0.20% $(1.48 \times 10^3/7.98 \times 10^5) \times 100$					

[1]nvd=no virus detected Exp = experiment

4.3.3. Detection of norovirus GI and GII from environmental swabs in simulation experiments

Environmental swabs of the food preparation area were taken straight after sandwich preparation across the six replicate simulation experiments, to identify the amount of norovirus deposited in the environment during sandwich preparation. The $\log_{10}$ cDNA copies of norovirus/swab were identified for norovirus GI positive swabs (Table 27) and norovirus GII positive swabs (Table 28). All swabs were taken by the same person and in duplicate throughout simulation experiments in order to standardise the swabbing technique (i.e. pressure applied, number of strokes, and area sampled). The size of the preparation tray, sandwich tray and lettuce bowl were kept consistent throughout the simulation experiments. Despite this, there was evidence of variation across some of the swabs taken in duplicate.

Table 27 norovirus Log_{10} cDNA copies/swab recovered from each environmental swabs to establish the level of norovirus contamination of the preparation area after each simulation experiment, and the average viral load transferred from two replicate swabs *= norovirus GI detected from one replicate only

Experiment number	Swab name	Log_{10} cDNA copies/swab	Total log_{10} cDNA copies from two replicates
Experiment 1	Preparation tray	3.19	3.35
	Preparation tray	2.84	
	Bowl	4.26	4.52
	Bowl	4.17	
	Sandwich tray	1.10	3.28
	Sandwich tray	3.28	
Experiment 3	Preparation tray	4.10	4.48
	Preparation tray	4.25	
	Bowl	3.74	3.99
	Bowl	3.65	
	Sandwich tray	nvd[1]	nvd[1]
	Sandwich tray	nvd	
Experiment 5	Preparation tray	nvd	5.66*
	Preparation tray	5.66	
	Bowl	nvd	3.69*
	Bowl	3.69	
	Sandwich tray	2.05	2.05*
	Sandwich tray	nvd	

Experiment number	Swab name	Log_{10} cDNA copies/swab	Total log_{10} cDNA copies from two replicates
Experiment 2	Preparation tray	2.14	2.61
	Preparation tray	2.43	
	Bowl	4.49	4.78
	Bowl	4.47	
	Sandwich tray	0.89	4.78
	Sandwich tray	4.78	
Experiment 4	Preparation tray	3.50	4.67
	Preparation tray	4.64	
	Bowl	1.29	1.34
	Bowl	0.34	
	Sandwich tray	nvd	nvd
	Sandwich tray	nvd	
Experiment 6	Preparation tray	1.84	2.14
	Preparation tray	1.85	
	Bowl	3.90	4.58
	Bowl	4.47	
	Sandwich tray	nvd	1.64*
	Sandwich tray	1.64	

[1]nvd=no virus detected.

Table 28 norovirus Log_{10} cDNA copies/swab recovered from each environmental swabs to establish the level of norovirus contamination of the preparation area after each simulation experiment, and the average viral load transferred from two replicate swabs *= norovirus GII was detected from one replicate only

Experiment number	Swab name	Log_{10} cDNA copies/swab	Total log_{10} cDNA copies from two replicates
Experiment 1	Preparation tray	2.00	2.00
	Preparation tray	nvd	
	Lettuce bowl	1.80	2.42
	Lettuce bowl	2.30	
	Sandwich tray	nvd	nvd
	Sandwich tray	nvd	
Experiment 3	Preparation tray	nvd	3.84
	Preparation tray	3.84	
	Lettuce bowl	3.26	3.26
	Lettuce bowl	nvd[1]	
	Sandwich tray	2.51	2.64
	Sandwich tray	2.06	
Experiment 5	Preparation tray	nvd	nvd
	Preparation tray	nvd	
	lettuce bowl	nvd	1.53*
	lettuce bowl	1.53	
	Sandwich tray	nvd	nvd
	Sandwich tray	nvd	

Experiment number	Swab name	Log_{10} cDNA copies/swab	Total log_{10} cDNA copies from two replicates
Experiment 2	Preparation tray	nvd	1.99*
	Preparation tray	1.99	
	Lettuce bowl	nvd	1.51*
	Lettuce bowl	1.51	
	Sandwich tray	nvd	nvd
	Sandwich tray	nvd	
Experiment 4	Preparation tray	2.70	2.97
	Preparation tray	2.63	
	Lettuce bowl	1.49	1.63
	Lettuce bowl	1.73	
	Sandwich tray	nvd	nvd
	Sandwich tray	nvd	
Experiment 6	Preparation tray	1.91	2.18
	Preparation tray	1.83	
	lettuce bowl	3.17	3.41
	lettuce bowl	3.04	
	Sandwich tray	3.58	3.58*
	Sandwich tray	nvd	

[1]nvd=no virus detected.

4.3.4. Summary of the total recovery and percentage recovery of log_{10} cDNA copies of norovirus from food handlers, food and the environment from six replicate simulation experiments

The total amount of norovirus recovered throughout sandwich making from each point of measure from food handler's gloved hands, food and the environment was summarised in Table 29 and Table 30. The percentage recovery was calculated using the average of two replicate inoculum specimens; the positive faecal material used to artificially contaminate the left hand of Food handler 1. It was important to consider the total amount of norovirus RNA recovered from all specimens. Throughout the six replicates norovirus GI simulation experiments the percentage of the inoculum recovered from food handler's hands, food and the environment ranged from 1% to 23% (Table 29) and for norovirus GII ranged from 1% to 32% (Table 30). In the simulation experiment with the most norovirus GI recovery, just under a third of the norovirus GI inoculum was recovered from food handlers, food and the environment, and in the simulation experiment with the most norovirus GII recovery slightly more than a fifth of the norovirus GI inoculum was recovered.

Table 29 presenting the viral loads of norovirus GI recovered from each data point across the six replicate simulations, the total amount of norovirus recovered from food handlers, food and the environment and the percentage recovery [total transferred/average inoculum x 100] [1]nvd= no virus detected

Sample Name	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
Inoculum	7.7	7.6	7.9	7.9	7.9	7.9
Inoculum	7.8	7.7	7.9	8.0	7.9	7.9
Average Inoculum (total log_{10} cDNA copies in inoculum/2)	7.7	7.7	7.9	8.0	7.9	7.9
Food handler 1 Left Hand (log_{10} cDNA copies/hand)	6.8	6.9	5.2	6.6	6.0	6.7
Food handler 1 Right Hand (log_{10} cDNA copies/hand)	5.7	6.5	5.0	6.1	5.3	6.7
Food handler 2 Left Hand (log_{10} cDNA copies/hand)	5.0	4.6	3.9	3.9	2.6	3.1
Food handler 2 Right Hand (log_{10} cDNA copies/hand)	4.3	2.7	3.4	3.4	3.6	3.8
Food handler 3 Left Hand (log_{10} cDNA copies/hand)	2.0	3.3	3.9	1.5	nvd	3.4
Food handler 3 Right Hand (log_{10} cDNA copies/ hand)	2.6	2.9	4.1	1.1	nvd	2.2
Sandwich (log_{10} cDNA copies/sandwich)	5.5	5.2	4.5	4.1	5.6	6.4
Sandwich (log_{10} cDNA copies/sandwich)	5.5	4.9	4.6	3.9	5.8	5.7
Preparation tray (log_{10} cDNA copies/swab)	3.2	2.1	4.1	3.5	nvd	1.8
Preparation tray (log_{10} cDNA copies/swab)	2.8	2.4	4.3	4.6	5.7	1.9
Bowl (log_{10} cDNA copies/swab)	4.3	4.5	3.7	1.3	nvd	3.9
Bowl (log_{10} cDNA copies/swab)	4.2	4.5	3.7	0.3	3.7	4.5
Sandwich tray (log_{10} cDNA copies/swab)	1.1	0.9	nvd	nvd	2.1	nvd
Sandwich tray (log_{10} cDNA copies/ swab)	3.3	4.8	nvd	nvd	nvd	1.6
Total percentage retained from hands, food and environment [total log_{10} cDNA copies /average inoculum x 100]	**14%**	**23%**	**1%**	**6%**	**3%**	**14%**

Table 30 presenting the viral loads of norovirus GII recovered from each data point across the six replicate simulations, the total amount of norovirus recovered from food handlers, food and the environment and the percentage recovery [total transferred/average inoculum x 100] [1]nvd= no virus detected

Sample Name	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
Inoculum	5.9	5.8	5.8	6.0	5.9	6.1
Inoculum	5.9	5.8	5.9	6.0	5.9	6.1
Average Inoculum (total log_{10} cDNA copies in inoculum/2)	5.9	5.8	5.9	6.0	5.9	6.1
Food handler 1 Left Hand (log_{10} cDNA copies/hand)	5.1	4.1	3.5	4.2	3.9	3.5
Food handler 1 Right Hand (log_{10} cDNA copies/hand)	5.1	2.7	2.2	3.9	3.3	3.2
Food handler 2 Left Hand (log_{10} cDNA copies/hand)	2.7	1.3	0.6	nvd	nvd	2.1
Food handler 2 Right Hand (log_{10} cDNA copies/hand)	2.9	1.5	nvd	3.5	2.1	nvd
Food handler 3 Left Hand (log_{10} cDNA copies/hand)	2.2	nvd	nvd	0.5	2.2	2.5
Food handler 3 Right Hand (log_{10} cDNA copies/ hand)	1.4	1.1	1.6	2.5	nvd	2.0
Sandwich (log_{10} cDNA copies/sandwich)	2.1	nvd	nvd	0.9	2.6	3.4
Sandwich (log_{10} cDNA copies/sandwich)	1.7	1.8	2.6	3.4	3.0	3.3
Preparation tray (log_{10} cDNA copies/swab)	2.0	nvd	nvd	2.7	nvd	1.9
Preparation tray (log_{10} cDNA copies/swab)	nvd	2.0	3.8	2.6	nvd	1.8
Bowl (log_{10} cDNA copies/swab)	1.8	nvd	3.3	1.5	nvd	3.2
Bowl (log_{10} cDNA copies/swab)	2.3	1.5	nvd	1.7	1.5	3.0
Sandwich tray (log_{10} cDNA copies/swab)	nvd	nvd	2.5	nvd	nvd	3.6
Sandwich tray (log_{10} cDNA copies/ swab)	nvd	nvd	2.1	nvd	nvd	nvd
Total percentage retained from hands, food and environment [total log_{10} cDNA copies /average inoculum x 100]	**32%**	**2%**	**2%**	**3%**	**1%**	**1%**

4.4. Characterisation of norovirus positive food handler's hands, the food matrices and environmental swabs during food production

4.4.1. Characterisation of norovirus GI and GII recovery from food handler's hands, food and environmental swabs by region C typing and dideoxynucleotide sequencing

Region C typing and characterisation by Sanger sequencing was conducted in these norovirus transfer simulation experiments in order to ensure that all positive specimens were from the same primary inoculum and demonstrate the use of characterisation of norovirus genomes recovered from different norovirus genotypes. However in real scenarios Sanger sequencing could be used to identify norovirus strains recovered from foodstuffs, and characterising these samples could be done to investigate outbreaks, or track transmission events in kitchens linked to outbreaks. Characterisation was conducted by amplification of Region C of the ORF 2 capsid region. Region C typing was conducted with the aim to establish the norovirus genotype in specimens (Kojima *et al.*, 2002, Gallimore *et al.*, 2005). All amplicons of expected size of 343bp by gel electrophoresis were purified and sequenced (Table 31 and Table 32). In the norovirus GI region C typing RT-PCR 16/16 inoculum, 6/12 food handler 1 samples were amplified and sequenced. No sequences were obtained from positive amplicons of food handler 2, food handler 3 or environmental swabs. A dendrogram of the dideoxynucleotide sequences from purified second round RT-PCR amplicons was constructed using algorithms in MegAlign (DNASTAR version 12.2) (Figure 34). All norovirus GI positive specimens that were sequenced were identified as 100% identical to each other. From this it can be interpreted that the sequences were of the same genotype, however in order to determine whether these sequences are of the same strain, analysis of Region C and the P domain is required.

Table 31 A table presenting specimens from norovirus GI food handler simulation experiments undergone region C typing, and second round RT-PCR amplicons from this typing have been resolved on a 1% agarose gel in the image below which shows wells 1-53 by gel electrophoresis. Positive results of the region C typing assay are demonstrated by a band at approximately 343bp demonstrated by the 100bp ladder (Invitrogen). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2

Well	Experiment	Name	Result	Well	Experiment	Name	Result
1	**1**	**Food Handler 1 Left Hand**	**+**	27	2	Bowl	-
2	**1**	**Food Handler 1 Right Hand**	**+**	28	2	Bowl	-
3	1	Food Handler 2 Left Hand	-	29	2	Preparation tray	-
4	1	Food Handler 2 Right Hand	-	30	2	Preparation tray	-
5	1	Food Handler 3 Left Hand	-	31	2	Sandwich tray	-
6	1	Food Handler 3 Right Hand	-	32	2	Sandwich	-
7	1	Sandwich	-	33	2	**Inoculum**	**+**
8	1	Sandwich	-	34	2	**Inoculum**	**+**
9	1	Bowl	-	35	2	**Inoculum**	**+**
10	1	Bowl	-	36	2	**Inoculum**	**+**
11	1	Preparation tray	-	37	3	Food Handler 1 Left Hand	-
12	1	Preparation tray	-	38	3	Food Handler 1 Right Hand	-
13	1	Sandwich tray	-	39	3	Food Handler 2 Left Hand	-
14	1	Sandwich tray	-	40	3	Food Handler 2 Right Hand	-
15	**1**	**Inoculum**	**+**	41	3	Food Handler 3 Left Hand	-
16	**1**	**Inoculum**	**+**	42	3	Food Handler 3 Right Hand	-
17	**1**	**Inoculum**	**+**	43	3	Sandwich	-
18	**2**	**Inoculum**	**+**	44	3	Sandwich	-
19	**2**	**Food Handler 1 Left Hand**	**+**	45	3	Bowl	-
20	2	Food Handler 1 Right Hand	-	46	3	Bowl	-
21	**2**	**Food Handler 2 Left Hand**	**+**	47	3	Preparation tray	-
22	2	Food Handler 2 Right Hand	-	48	3	Preparation tray	-
23	2	Food Handler 3 Left Hand	-	50	3	Food Handler 2 Left Hand	-
24	2	Food Handler 3 Right Hand	-	51	3	Food Handler 3 Right Hand	-
25	2	Sandwich	-	52	3	Sandwich	-
26	2	Sandwich	-	53	3	Bowl	-

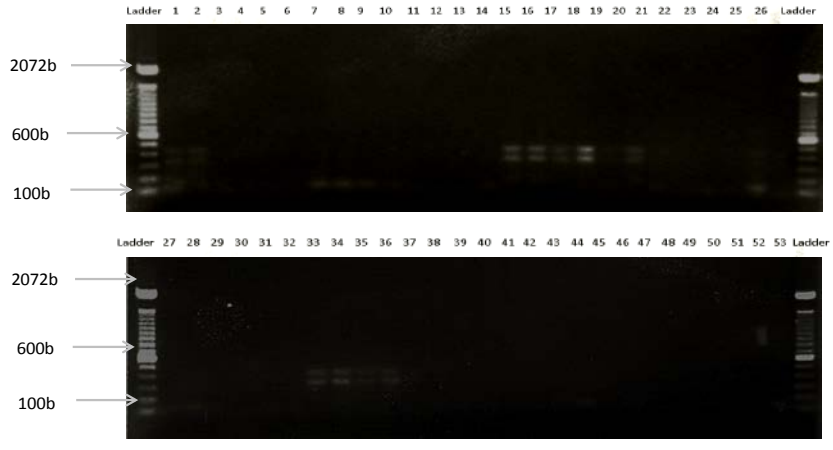
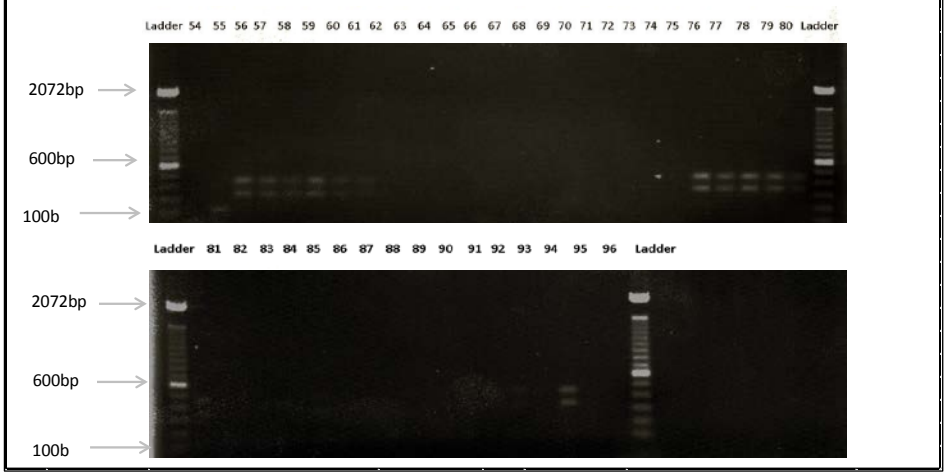


Table 32 A table presenting specimens from norovirus GI food handler simulation experiments undergone region C typing, and second round RT-PCR amplicons from this typing have been resolved on a 1% agarose gel in the image below which shows wells 54-96 by gel electrophoresis. Positive results of the region C typing assay are demonstrated by a band at approximately 343bp demonstrated by the 100bp ladder (Invitrogen). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2

Well	Experiment	Name	Result	Well	Experiment	Name	Result
54	3	Bowl	-	81	5	Food Handler 2 Right Hand	-
55	**3**	**Inoculum**	**+**	82	5	Food Handler 3 Left Hand	-
56	**3**	**Inoculum**	**+**	83	5	Food Handler 3 Right Hand	-
57	**3**	**Inoculum**	**+**	84	5	Sandwich	-
58	**3**	**Inoculum**	**+**	85	5	Sandwich	-
59	**4**	**Food Handler 1 Left Hand**	**+**	86	5	Bowl	-
60	**4**	**Food Handler 1 Right Hand**	**+**	87	5	Bowl	-
61	4	Food Handler 2 Left Hand	-	88	5	Preparation tray	-
62	4	Food Handler 2 Right Hand	-	89	5	Preparation tray	-
63	4	Food Handler 3 Left Hand	-	90	6	Food Handler 1 Left Hand	-
64	4	Food Handler 3 Right Hand	-	91	6	Food Handler 2 Right Hand	-
65	4	Sandwich	-	92	6	Food Handler 1 Left Hand	-
66	4	Sandwich	-	**93**	**6**	**Food Handler 1 Right Hand**	**+**
67	4	Bowl	-	94	6	Food Handler 2 Left Hand	-
68	4	Bowl	-	95	6	Inoculum	
69	4	Preparation tray	-	96	6	Inoculum	
70	4	Preparation tray	-				
71	4	Food Handler 1 Right Hand	-				
72	4	Preparation tray	-				
73	4	Sandwich tray	-				
74	**4**	**Inoculum**	**+**				
75	**4**	**Inoculum**	**+**				
76	**4**	**Inoculum**	**+**				
77	**4**	**Inoculum**	**+**				
78	**5**	**Food Handler 1 Left Hand**	**+**				
79	**5**	**Food Handler 1 Right Hand**	**+**				
80	5	Food Handler 2 Left Hand	-				



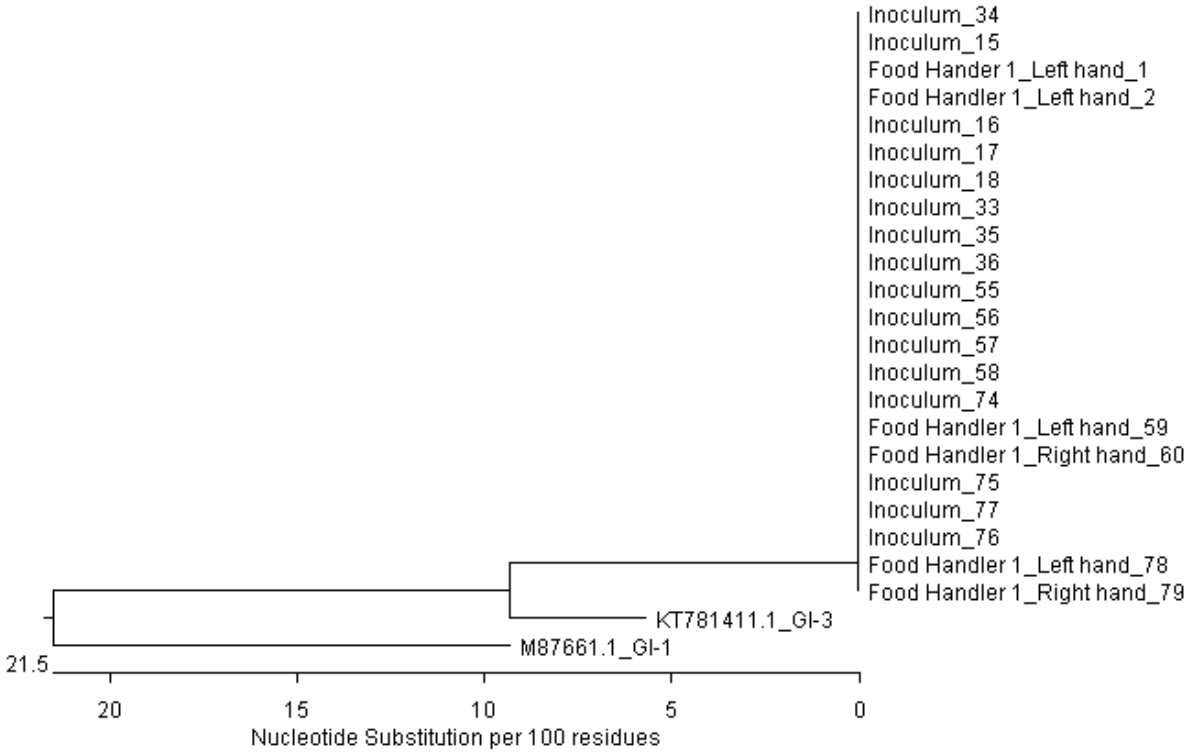


Figure 34 A dendrogram of dideoxynucleotide sequences obtained from purified second round RT-PCR amplicons in Table 31 and Table 32 (well number_experiment_sample name) to confirm genetic homogeneity of norovirus transferred from food handlers, food and environmental swabs. Clustal W analysis aligned using algorithms in MegAlign (DNASTAR version 12.2) and based on phylogenetic relatedness to the Norwalk GI-I reference strain accession number M87661.1 (GenBank) and a GI-3 reference strain accession number KT781411.1.

For norovirus GII region C typing RT-PCR, 9/12 inoculum; 3/12 foods; 6/12 Food handler 1; 6/12 Food handler 2; 3/12 Food handler 3 and 11/36 swabs were sequenced (Table 33 and Table 34). All of the positive specimens from the simulation experiments were 100% identical to each other and 97% identical to the Lordsdale GII.4 reference strain accession number X87655.1 (Figure35). Further characterisation by amplification of the P2 domain was required in order to identify the sequences were truly identical as the region C typing does not provide sufficient resolution to establish the difference between norovirus strains. Not all sequences of specimens were able to be characterised by region C, demonstrating the difficulty in obtaining sequencing information for complex analytes and positive specimens with a range of viral loads. Furthermore the region C genotyping method is a published assay designed and validated for genotyping faecal samples, therefore the non-specific binding identified by the multiple bands on the norovirus GII gels suggest the assay may need optimising for use with food and environmental samples.

Table 33 A table presenting specimens from norovirus GII food handler simulation experiments undergone region C typing, and second round RT-PCR amplicons from this typing have been resolved on a 1% agarose gel which shows well 1-48 in the image below by gel electrophoresis. Positive results of the region C typing assay are demonstrated by a band at approximately 343bp demonstrated by the 100bp ladder (Invitrogen). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2.

Well	Experiment	Name	Result	Well	Experiment	Name	Result
1	1	Sandwich	+ n/s	25	**2**	**Bowl**	**+**
2	1	Food Handler 1 Left Hand	+ n/s	26	2	Preparation tray	+ n/s
3	1	Food Handler 1 Right Hand	-	27	2	Preparation tray	+ n/s
4	**1**	**Food Handler 2 Left Hand**	**+**	28	**2**	**Inoculum**	**+**
5	**1**	**Food Handler 2 Right Hand**	**+**	29	**2**	**Inoculum**	**+**
6	1	Food Handler 3 Left Hand	-	30	**3**	**Food Handler 1 Left Hand**	**+**
7	**1**	**Food Handler 3 Right Hand**	**+**	31	**3**	**Food Handler 1 Right Hand**	**+**
8	**1**	**Sandwich**	**+**	32	**3**	**Food Handler 2 Left Hand**	**+**
9	1	Preparation tray	+ n/s	33	**3**	**Food Handler 2 Right Hand**	**+**
10	1	Preparation tray	-	34	**3**	**Food Handler 3 Right Hand**	**+**
11	1	Bowl	-	35	**3**	**Sandwich**	**+**
12	1	Bowl	+ n/s	36	**3**	**Bowl**	**+**
13	1	Negative Inoculum	-	37	**3**	**Bowl**	**+**
14	1	Negative Inoculum	-	38	**3**	**Preparation tray**	**+**
15	2	Preparation tray	+ n/s	39	**3**	**Preparation tray**	**+**
16	**2**	**Food Handler 1 Left Hand**	**+**	40	3	Sandwich tray	+ n/s
17	**2**	**Food Handler 1 Right Hand**	**+**	41	3	Preparation tray	+ n/s
18	2	Food Handler 2 Left Hand	+ n/s	42	**3**	**Inoculum**	**+**
19	**2**	**Food Handler 2 Right Hand**	**+**	43	**3**	**Inoculum**	**+**
20	2	Food Handler 3 Right Hand	+ n/s	44	4	Food Handler 1 Left Hand	+ n/s
21	2	Food Handler 3 Left Hand	+ n/s	45	4	Food Handler 1 Right Hand	+ n/s
22	2	Sandwich	+ n/s	46	4	Food Handler 2 Left Hand	+ n/s
23	**2**	**Sandwich**	**+**	47	4	Food Handler 2 Right Hand	+ n/s
24	2	Bowl	+ n/s	48	4	Food Handler 3 Left Hand	+ n/s

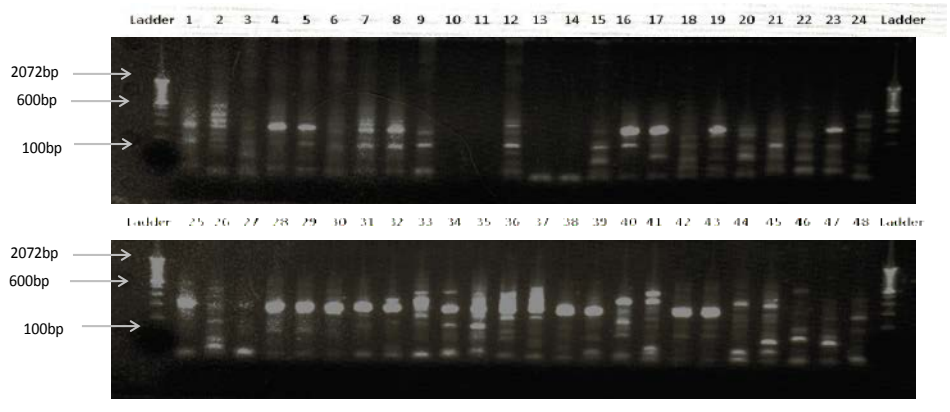
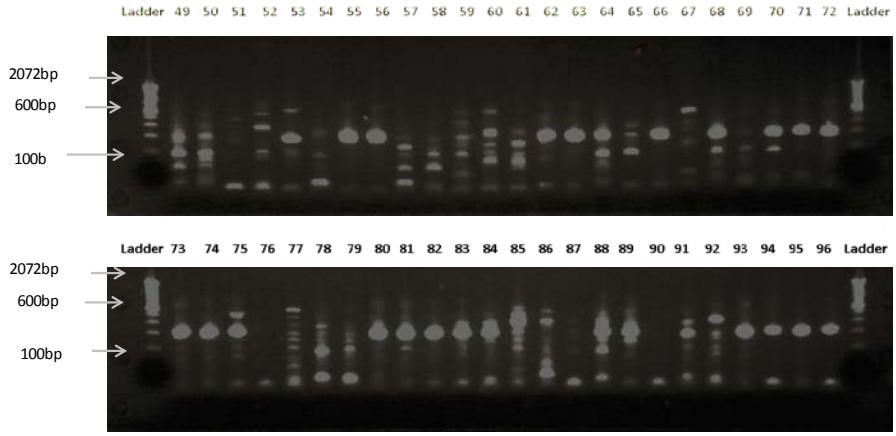


Table 34 A table presenting specimens from norovirus GII food handler simulation experiments undergone region C typing, and second round RT-PCR amplicons from this typing have been resolved on a 1% agarose gel in the image below which shows wells 49-96 by gel electrophoresis. Positive results of the region C typing assay are demonstrated by a band at approximately 343bp demonstrated by the 100bp ladder (Invitrogen). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2.

Well	Experiment	Name	Result	Well	Experiment	Name	Result
49	4	Food Handler 3 Right Hand	+ n/s	**73**	**4**	**Inoculum**	**+**
50	4	Sandwich	+ n/s	**74**	**5**	**Food Handler 1 Left Hand**	**+**
51	4	Sandwich	-	**75**	**5**	**Food Handler 1 Right Hand**	**+**
52	4	Bowl	+ n/s	76	**5**	Food Handler 2 Right Hand	-
53	**4**	**Bowl**	**+**	77	**5**	Food Handler 3 Left Hand	+ n/s
54	4	Sandwich tray	+ n/s	78	**5**	Sandwich	+ n/s
55	**4**	**Sandwich tray**	**+**	79	**5**	Bowl	+ n/s
56	**4**	**Preparation tray**	**+**	**80**	**5**	**Inoculum**	**+**
57	4	Preparation tray	+ n/s	**81**	**5**	**Inoculum**	**+**
58	4	Food Handler 1 Left Hand	+ n/s	**82**	**6**	**Food Handler 1 Left Hand**	**+**
59	4	Food Handler 1 Right Hand	+ n/s	**83**	**6**	**Food Handler 1 Right Hand**	**+**
60	4	Food Handler 2 Left Hand	+ n/s	**84**	**6**	**Food Handler 2 Left Hand**	**+**
61	4	Food Handler 2 Right Hand	+ n/s	**85**	**6**	**Food Handler 3 Left Hand**	**+**
62	**4**	**Food Handler 3 Left Hand**	**+**	86	6	Food Handler 3 Right Hand	+ n/s
63	**4**	**Food Handler 3 Right Hand**	**+**	87	6	Sandwich	-
64	**4**	**Sandwich**	**+**	**88**	**6**	**Sandwich**	**+**
65	4	Sandwich	+ n/s	**89**	**6**	**Bowl**	**+**
66	**4**	**Bowl**	**+**	90	6	Bowl	-
67	4	Bowl	+ n/s	91	6	Preparation tray	+ n/s
68	**5**	**Sandwich tray**	**+**	92	6	Preparation tray	+ n/s
69	5	Sandwich Tray	-	**93**	**6**	**Inoculum**	**+**
70	**5**	**Preparation tray**	**+**	**94**	**6**	**Inoculum**	**+**
71	**5**	**Preparation tray**	**+**	**95**		**PCR Positive control**	**+**
72	**4**	**Inoculum**	**+**	**96**		**PCR Positive control**	**+**



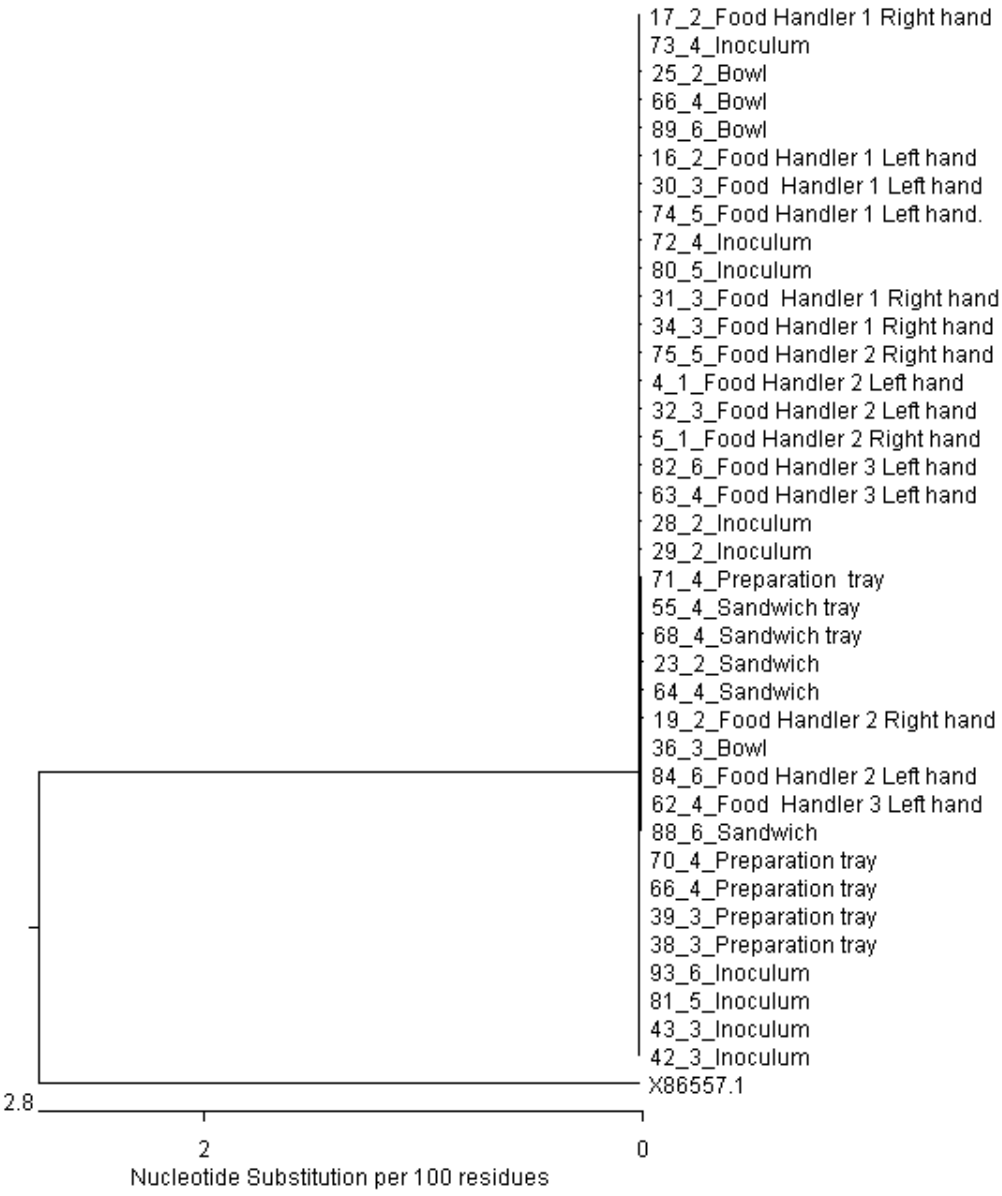


Figure 35 A dendrogram of dideoxynucleotide sequences obtained from purified second round RT-PCR amplicons in Table 33 and Table 34 (well number_experiment_sample name) to confirm genetic homogeneity of norovirus transferred from food handlers, food and environmental swabs. Clustal W analysis aligned using algorithms in MegAlign (DNASTAR version 12.2) and based on phylogenetic relatedness to the Lordsdale GII.4 reference strain accession number X86557.1 (GenBank).

4.4.2. Characterisation of norovirus GI and GII recovery from Food Handlers hand, food and environmental swabs by P2 intra-typing assay and dideoxynucleotide sequencing

Further characterisation of all positive specimens was conducted through dideoxynucleotide sequencing of the P2 domain with the aim to identify the number of SNPs within this region, so that all positive specimens could be linked to the same norovirus strain and part of the same simulated transmission event. Amplification of the P2 domain by PCR for all GI positive specimens failed to give any bands by gel electrophoresis, therefore no sequence data was obtained from these specimens. Amplification of the P2 domain by PCR for the GII positive specimens were identified by an expected band size of 512bp when resolved on a 1% agarose gel by gel electrophoresis. Sequences were obtained from 7/13 inoculum controls; 4/35 food handlers and 1/47 swabs (Table 35 and Table 36). Clustal W multiple alignment using algorithms in MegAlign was conducted to identified phylogenetic relatedness (DNASTAR version 12.2) (Figure 36). Specimens in well number 49, 51, 59 & 67 failed to give full length quality trace data from amino acid 279 to 405 (Prasad *et al.,* 1999), and for these reasons were eliminated from the dendrogram analysis. There were no SNPs in the P2 domain amplicons indicating only one strain was present, and that these specimens were from the same simulated transmission event. The P2 domain PCR and sequencing data demonstrated these identical strains clustered closet to DQ078794 Hunter strain (Bull *et al*., 2006).

Table 35 A table presenting specimens from norovirus GII food handler simulation experiments undergone P2 intra-genotyping, and second round RT-PCR amplicons from this typing have been resolved on a 1% agarose gel in the image below by gel electrophoresis. Positive results of the P2 intra-genotyping assay are demonstrated by a band at approximately 512bp demonstrated by the 100bp ladder (Invitrogen). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2 (n/s=non specific).

Well	Experiment	Name	Result	Well	Experimen	Name	Result
1	1	Sandwich	-	25	2	Preparation tray	-
2	1	Food Handler 1 Left Hand	-	**26**	**2**	**Inoculum**	**+**
3	**1**	**Food Handler 1 Right Hand**	**+**	**27**	**2**	**Inoculum**	**+**
4	1	Food Handler 2 Left Hand	-	28	2	Food Handler 1 Left Hand	-
5	1	Food Handler 2 Right Hand	-	29	2	Food Handler 1 Right Hand	-
6	1	Food Handler 3 Left Hand	-	30	3	Food Handler 2 Left Hand	-
7	1	Food Handler 3 Right Hand	-	31	3	Food Handler 2 Right Hand	-
8	1	Sandwich	-	32	3	Food Handler 3 Right Hand	-
9	1	Preparation tray	-	33	3	Sandwich	-
10	1	Preparation tray	-	34	3	Bowl	-
11	1	Bowl	-	35	3	Bowl	-
12	1	Bowl	-	36	3	Preparation tray	-
13	1	Preparation tray	-	37	3	Preparation tray	-
14	**2**	**Food Handler 1 Left Hand**	**+**	38	3	Sandwich tray	-
15	2	Food Handler 1 Right Hand	-	**39**	**3**	**Preparation tray**	**+**
16	2	Food Handler 2 Left Hand	-	**40**	**3**	**Inoculum**	**+**
17	2	Food Handler 2 Right Hand	-	**41**	**3**	**Inoculum**	**+**
18	2	Food Handler 3 Right Hand	-	42	3	Sandwich tray	-
19	2	Food Handler 3 Left Hand	-	43	3	Sandwich tray	-
20	2	Sandwich	-	**44**	**4**	**Food Handler 1 Left Hand**	**+**
21	2	Sandwich	-	**45**	**4**	**Food Handler 1 Right Hand**	**+**
22	2	Bowl	-	46	4	Food Handler 2 Left Hand	-
23	2	Bowl	-	47	4	Food Handler 2 Right Hand	-
24	2	Preparation tray	-	48	4	Food Handler 3 Right Hand	-

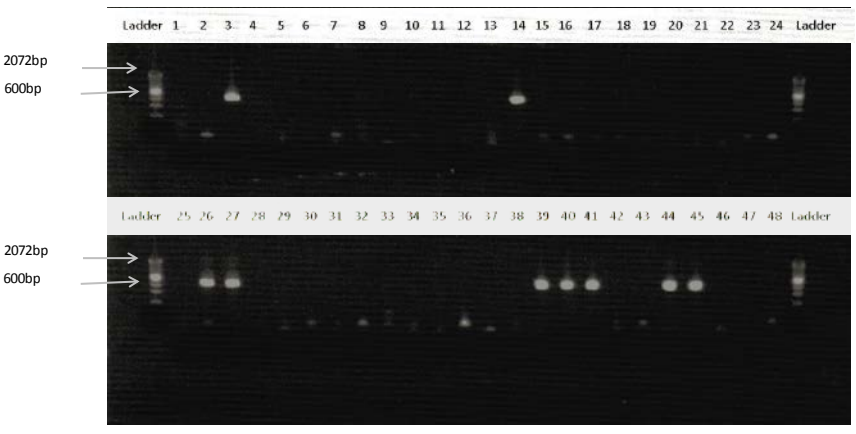
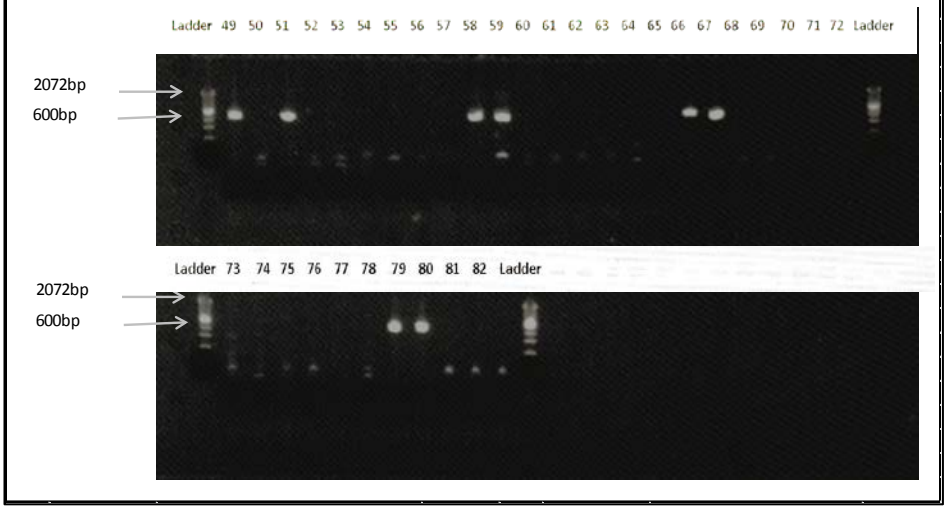


Table 36 A table showing the second round RT-PCR amplicons from wells 49-82 of norovirus in GII food handler experiments and the 100bp ladder (Invitrogen), resolved on a 1% agarose gel by gel electrophoresis (Invitrogen). Positive results of the intra-genotyping RT-PCR assay are demonstrated by a band at approximately 512bp (n/s=non specific). Pink= Food handler 1, Green=Food Handler 2, Pale blue= Food handler 3, Dark blue=sandwich half 1, Red= Sandwich half 2.

Well	Experiment	Name	Result	Well	Experiment	Name	Result
49	**4**	**Sandwich**	**+**	73	6	Sandwich	-
50	4	Sandwich	-	74	6	Bowl	-
51	**4**	**Bowl**	**+**	75	6	Bowl	-
52	4	Bowl	-	76	6	Preparation tray	-
53	4	Sandwich tray	-	77	6	Preparation tray	-
54	4	Sandwich tray	-	78	6	Inoculum	-
55	4	Preparation tray	-	79	**6**	**Inoculum**	**+**
56	4	Preparation tray	-	80	**6**	**Inoculum**	**+**
57	4	Inoculum	-	81	1	Inoculum	-
58	**4**	**Inoculum**	**+**	82	1	Inoculum	-
59	**5**	**Food Handler 1 Left Hand**	**+**	83			
60	5	Food Handler 1 Right Hand	-	84			
61	**5**	Food Handler 2 Right Hand	-	85			
62	5	Food Handler 3 Left Hand	-	86			
63	**5**	Sandwich	-	87			
64	5	Bowl	-	88			
65	5	Inoculum	-	89			
66	**5**	**Inoculum**	**+**	90			
67	**6**	**Food Handler 1 Left Hand**	**+**	91			
68	6	Food Handler 1 Right Hand	-	92			
69	6	Food Handler 2 Right Hand	-	93			
70	6	Food Handler 3 Left Hand	-	94			
71	6	Food Handler 3 Right Hand	-	95			
72	6	Sandwich	-	96			



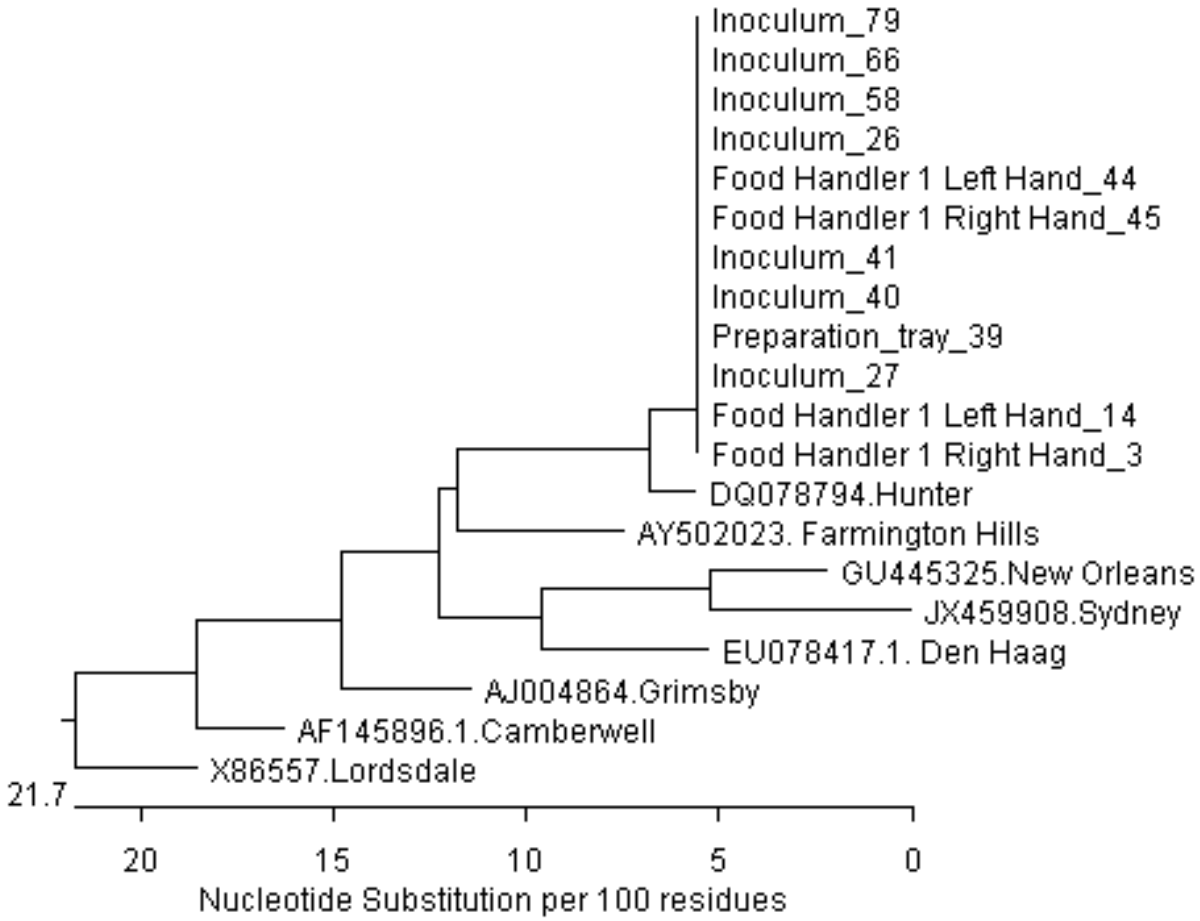


Figure 36 A dendrogram of dideoxynucleotide sequences obtained from purified second round RT-PCR amplicons in Table 33 and Table 34 (well number_experiment_sample name) to confirm genetic homogeneity of norovirus transferred from food handlers, food and environmental swabs. Clustal W analysis aligned using algorithms in MegAlign (DNASTAR version 12.2) and based on phylogenetic relatedness to the Lordsdale GII.4 reference strain accession number X86557.1 (GenBank).

4.5. Discussion

4.5.1. Establishing a protocol to identify food handler contamination

Ready to eat foods present the greatest risk to consumers because they do not require further processing and are ready for direct human consumption (HPA, 2009). Furthermore, soft fruits and salad vegetables are at risk of contamination during production, and the more intensively handled the RTE food the greater the risk of becoming contaminated if prepared by an infected food handler. There are many types of RTE food that could have been selected in the simulation experiments, but preparing a sandwich was selected as this process requires substantial handling and usually involves a number of different RTE ingredients. Sandwiches have also been identified as they have been implicated in a number of foodbone outbreaks for viral contamination (Baert et al., 2009, Parashar et al., 1998, Daniels et al., 2000b).

The simulation of the sandwich making process was divided into tasks conducted by different food handlers. In a survey completed by caterers, 38% of respondents thought it safe for restaurant workers infected with norovirus to handle packaged food, equipment and utensils (Kosa *et al.*, 2014). Therefore, packaging the sandwich was included in the simulation design as an aspect of sandwich making. Division of the final sandwich prior to packaging was also included in the process however this step was done by hand in order to reduce risks to the food handler volunteers. It was acknowledged that utensils would typically be used during production, and may act as a vehicle to transfer norovirus from the hands of one food handler to the next or may result in the cross contamination of ingredients. This was identified in an artificial contamination study by Wang *et al.* (2013) in which the cross contamination of murine norovirus occurred in up to seven different fresh produce that were successively prepared using a single contaminated knife.

In order to establish a protocol that best simulates food handler contamination, the HSE publication on commonly missed areas during hand washing was referred to. It identified three commonly missed areas during hand washing as the palm of the hand, the fingertips and along the fingers. Other study designs on artificial hand contamination have chosen to artificially contaminate fingertips with a single spot of virus in their studies; to represent poor hand hygiene (Ansari *et al.,* 1988, Bidawid *et al.,* 2000a, Rönnqvist *et al.,* 2014). Consistent with other study designs, this method was chosen to best represent artificial contamination. This was due to the fact that it was the most efficient method of virus transfer from the left inoculated to the right non-inoculated hand and as identified in Table 21, resulted in the greatest percentage of norovirus being retained on both hands.

4.5.2. Inoculums used for hand contamination in simulation experiments

Faecal specimens used in hand contamination experiments were chosen to represent true hand contamination, and are more readily available specimens in comparison to cell culture material, as a cell culture model is not routinely applicable for norovirus yet. The high viral load in faecal specimens was adapted for hand contamination by distributing a small volume onto the non-dominant hand of food handler volunteers. The two inoculums used in hand contamination both contained high viral loads, as quantitative data on norovirus transfer was not available from the literature to help guide the inoculum to use. Therefore in future work, ten-fold dilutions of the inoculum would need to be conducted to see how lower viral loads transfer during food preparation, and what percentage can be recovered.

Inoculums used in this study, to contaminate the fingertips of food handler's gloves were representative of the viral loads that might be present in infected individual's faecal

specimen. As indicated in a study by Ozawa *et al.* (2007), in which 2.79×10^7 cDNA copies per gram of faeces of norovirus GI and 3.81×10^8 cDNA copies per gram of faeces of norovirus GII were identified in some asymptomatic food handlers. Teunis *et al.* (2015) reported on average 10^5–10^9 cDNA copies per gram of faeces in asymptomatic and symptomatic food handlers during peak infection. Atmar *et al.* (2008) reported norovirus shedding of 95×10^9 cDNA copies per gram of faeces.

4.5.3. Determining whether norovirus remains detectable by real time RTqPCR throughout food production

A reduction in the amount of norovirus was detected across the tasks as they were performed in the simulation experiments. This was first demonstrated in the preliminary experiment in which an inoculum of 6.2 (SD ± 0.01) $\log_{10}$ cDNA copies of norovirus per µl of inoculum was used to inoculate the hand, and on completion of the food preparation simulation the amount recovered did not total the original inoculum value. As shown in Table 22, there was a clear decrease in percentage recovery across the experiment. This indicates that virus must be lost through the food production process and through sample processing methods from the sample preparation to the extraction and PCR. In Chapter 3 it was identified that acidic pH increased norovirus capture by PGM conjugated magnetic beads, however it is possible that this pH may have aided virus absorption onto other surfaces during processing, such as the gloves, food and to the separator 400 blender bag. The data in Chapter 3 identified that the capture of norovirus from samples was not 100% efficient. Loss of virus to the plastic of the 50ml tubes could have occurred during incubation of the samples with PGM conjugated magnetic beads. Loss of norovirus could have continued throughout the extraction and molecular process however; sensitivity constraints of the extraction and detection methods were explored in Chapter 3 to reduce

virus loss at this stage. Overall, small losses at each stage of processing contribute to a cumulative level of viral loss by the end of sample processing.

4.5.4. Virus recovery from the hands

Despite the loss of norovirus throughout the testing process, quantitative data demonstrated recovery during the experiment specifically from the hands. An input inoculum containing 5.9 (SD ± 0.1) $\log_{10}$ cDNA copies per µl or 8.0 x 10^5 cDNA copies per µl of norovirus GII inoculum transferred to at least two other food handlers via the food ingredients handled. This also occurred in the simulation experiments in which 7.8 (SD ± 0.1) $\log_{10}$ cDNA copies per µl or 6.9 x 10^7 cDNA copies per µl of inoculum was used to inoculate the glove. Gloves were worn during these experiments, and can be used in food preparation, however it was identified in one observational study of 321 food workers, that when gloves are worn, hand washing was less likely to take place (Green *et al.,* 2007). Therefore this study demonstrates the importance of hand hygiene, and that those food handlers who do not practice good hand hygiene are at risk of transferring norovirus further or potentially becoming infected themselves. Overall Gloves that are cumbersome or ill-fitting can reduce dexterity which may deter food handlers from wearing them, increasing the risk of manual handling through bare hand contact resulting in an increased risk in food contamination. The catering gloves used throughout the simulation experiments were made from vinyl and it was noted that for those individuals in which gloves did not appropriately fit that the integrity of the gloves were poor and quite often tore during the process of putting on the gloves. Although PPE such as gloves are worn in order to provide a basic level of protection, it is still possible that gloves could become contaminated during food preparation with a contaminated food item, which could result

in self contamination during the process of removing the gloves. Therefore, it is important that catering establishments provide relevant training in wearing and removal of gloves.

4.5.5. Virus recovery from food

The average amount of norovirus recovered from a sandwich piece from the six replicates was 3.2 $\log_{10}$ cDNA (SD ±0.76) copies per sandwich for norovirus GII and 6.7 $\log_{10}$ cDNA (SD ±0.79) copies per sandwich for norovirus GI. Although expressed as a percentage this average equated to 0.2% and 1.2% transfer of the inoculum onto prepared sandwiches, these viral loads certainly present a risk to the consumer, particularly as the infectious dose for norovirus is believed to be less than 100 virus particles (Teunis *et al.*, 2008). Studies using viruses that can be grown in cell culture have reported similar transfer rates to hands and food as those reported in this study. Plaque assays to assess virus recovery and infectivity with rotavirus (Ansari *et al.*, 1988) and hepatitis A (Bidawid et al., 2000a) have been conducted through artificial inoculation of the fingertips of volunteers. Ansari *et al*. (1988) used a 10% faecal suspension containing 2 x 10^4 to 8 x 10^4 PFU of rotavirus, inoculated on fingertips transferred to a clean disk by applying 1kg per cm^2 of pressure, which resulted in recovery between 1.8% to 16.1%. In a contaminated hand to clean hand transfer experiment, recovery between 2.8% to 6.6% of infectious virus occurred. In a study by (Bidawid et al., 2000b) touching lettuce with artificially contaminated fingers containing 3 x 10^5 PFU for 10 seconds at a pressure of 0.2 to 0.4 kg per cm^2 resulted in a transfer rate of 9.2% infectious virus. Due to a lack of a routinely available cell culture model, infectious norovirus could not be determined. Despite these two studies presenting the transfer rates of two different viruses between food and surfaces, they align with the 11.6% observed in section 4.2.3 and the 9.6% recovery from gloved hands observed in section 4.3.1 after shredding lettuce in this thesis. Furthermore, Rönnqvist *et al.* (2014) inoculated

donor surfaces such as both right and left latex gloved hands with 3.5 $\log_{10}$ PCR units of norovirus and identified 33% ± 10% transfer efficiencies to latex gloves, 27% ± 8% to plastic and cucumber 22% ± 7% to cucumber.

4.5.6. Virus recovery to the environment

Loss of virus after each task could have been a result of norovirus onto contact surfaces such as the food preparation area. This was recognised during the development of the simulation protocol and environmental swabbing was introduced to help monitor this transfer. Environmental surfaces including the food preparation tray, sandwich tray and lettuce bowl were tested once the final protocol was established to identify the percentage of norovirus recovered beyond that on the food handler's hands and on food. A validated protocol for the detection of norovirus from environmental surfaces already exists, which could be applied during the study (Boom *et al.,* 1999; ISO/TS 15216; 2013 and PHE SOP V-5324, 2016). However in the final protocol, processing the gloves, food and environmental swabs for molecular testing is likely to have influenced the level of norovirus detection. A loss of viral load is likely to also occur during in the processing of the environmental swabs. In a study on the recovery of bacterial targets from swabs identified that vortex mixing swab tips for 15 seconds, is sufficient to obtain around 50% microbial recovery (Satyada and Sandle, 2016). An already established protocol was carried out regarding the type of swab used to detect norovirus, it could be that an alternative material to the viscose swab used in these experiments to measure hygiene, may provide better recovery efficiency. However viscose swabs were used instead of cotton swabs stated in ISO 15216 (2013), as cotton has been found interfere with PCR detection when using fluorescent dyes in real time PCR detection (CDC, 2002). Depending on the context of the outbreak investigation, swabs may be used in immediate outbreak investigations to check levels post cleaning or for surveillance. In circumstances such as deep cleaning of the premises as part of infection

control after an outbreak, the hygiene is likely to be better that that seen during normal operation of the catering premises (Smith *et al.*, 2012). In order to standardise the swabbing technique, the same person was used to swab environmental surfaces. Although swabs were taken in duplicate, the swabbing technique employed throughout was to hold both viscose swabs in one hand and move them in large strokes over the food preparation area. It is possible that in some instances where the same environmental surface produced two different viral loads from the duplicate swabs; i.e. the majority of the contamination was collected by one viscose swab more than the other, despite the fact swabs were taken in parallel. The swabbing technique was not optimised in this process, as the aim of the study was to provide a protocol to quantify and monitor norovirus recovery between food handler hands and food, which can be used alongside environmental surveillance through swabbing of premises to measure the efficiency of cleaning procedures and the general level of hygiene in catering establishments.

4.5.7. Anonymizing inoculums in food simulation experiments

Volunteers were not aware whether their left hand was being contaminated with inoculum or a 200µl of water as a mock. This was anonymised to encourage consistent food handling behaviour during their preparation of the sandwich. Human handling behaviour could not be controlled in the experiment, but it is something that will have impacted on the results and can be seen through the variation in viral loads recovered across the six replicates of each task and across the calculated percentage recovery (Figure 31 and Figure 32). Although viral loads identified from food handlers, food and the environment were presented separately, they were acknowledged as being from the same primary inoculum or source. Therefore, the total was calculated so that the percentage recovery of norovirus found in each of the six replicate simulations could be compared to the average viral load in the input inoculum. The percentage recovery ranged from 1% to 23% for norovirus GI and

1% to 32% from norovirus GII. Although these percentage recoveries are low, this is within the range reported by Bidawid *et al.* (2000a) in hepatitis A contamination experiments and by Ansari *et al.* (1988) for rotavirus. It must also be recognised that comparison is hindered because different protocols, elution methods, different viruses and detection methods are used in different published studies.

4.5.8. Characterisation of specimens from food handlers, food and the environment of the food preparation area during food production

All norovirus positive specimens identified by real time RT-PCR in the simulation experiments were characterised to demonstrate that they were from the same primary inoculum, this provided an insight into the potential use of genotyping methodologies in outbreak investigations for the purpose of tracking transmission in kitchens, from food, food handlers hands and fomites, where low levels of material might be identified. There are two regions commonly used to characterise norovirus strains, these are the polymerase (ORF1) or the capsid (ORF 2). Previously the ORF 1 was considered the most conserved domain (Green *et al*., 1993 ; Ando *et al*., 1995), and broad primer sets were designed to amplify this region (Green *et al.,* 1993, Ando *et al.,* 1995, Vinje and Koopmans, 1996, Le Guyader *et al.,* 1996). However due to the genetic diversity of norovirus, the ORF 2 section was amplified in a wider range of norovirus genotypes using Region C primer sets (Kojima *et al.*, 2002) in order to identify norovirus genotype; before characterisation could be conducted using genotype specific primer sets which target the P2 domain. The non-specific binding identified by the multiple bands observed on the gels in the amplification of norovirus GII by Region C PCR suggest the assay may need optimising for use with food and environmental samples, as the assay was originally designed for amplification of norovirus from faeces Amplification and characterisation of the P2 domain was conducted

as amplification of Region C of the ORF 2 provides insufficient information to be used in transmission events (Lopman, 2006). The P2 domain is expected to be 100% identical in norovirus outbreaks caused by food-handler contamination by an individual shedding norovirus. Analysis of the genes encoding the genotype specific P2 domain can be used to identify transmission events in acute cases, as no single nucleotide polymorphism (SNP) variation in this region was the result of specimens from the same transmission event (Xerry *et al.*, 2008). Although according to Sukhrie et al. (2013) P2 domain variation increases with duration of virus shedding, and it is possible that depending on the time of collection of samples, a degree of strain variability may occur. Attempts were made to amplify the P2 domain in all samples from the norovirus GI simulation experiments, but all specimens failed to amplify at the PCR stage including positive PCR controls. Actions to mitigate this failure included the use of single use aliquots of reagents to reduce the risk of degradation through repetitive freeze thawing. The assay used was well established and had been designed, validated and implemented in clinical specimens that contain higher viral loads. It may therefore be the case that this assay was not suitable for food and environmental specimens due to its sensitivity, or as a result sample associated interference that may have occurred within the PCR. The sensitivity of this genotyping PCR may need to be optimised to detect lower viral loads if this method is to be used for the detection of viruses in foods in future foodborne outbreaks. Attempts were made to amplify the P2 domain in samples from the norovirus GI simulation experiments, but all specimens failed to amplify at the PCR stage including positive PCR controls. Actions to mitigate this failure included the use of single use aliquots of reagents to reduce the risk of degradation through repetitive freeze thawing. The assay used was well established and had been designed, validated and implemented in clinical specimens that contain higher viral loads. It may therefore be the case that this assay was not suitable for food and

environmental specimens due to its sensitivity, or as a result sample associated interference may have occurred within the PCR. In future work the sensitivity of this genotyping PCR may need to be optimised to detect lower viral loads, and provide enough amplified genomic material to obtain sequence information.

The P2 region of the norovirus GII simulation experiment specimens were analysed, and as defined by Prasad *et al.* (1999) amino acid positions ranging from 279 to 405 specifically for GII-4 intra-genotyping were analysed, and 100% homogeneity was observed in 12 full length sequences obtained (Figure 36). The P2 domain of norovirus GII was not detected on sandwiches. Therefore, sensitivity of the testing method is critical for testing food.

4.5.9. Overall discussion and future work

The main limitation of processing all food matrices by surface washing is that it may not be appropriate for those foods that may be internally contaminated, such as molluscan bivalves. Molluscan bivalves concentrate viruses within their digestive tissues during filter feeding (Torok, 2013). Norovirus contamination of molluscan bivalves during production or harvest in sewage containing water has been identified in many studies (Le Guyader *et al.,* 2006, Escudero *et al.,* 2012, Campos *et al.,* 2016, Boxman *et al.,* 2016). For this reason, the method described in ISO 15216 (2013), in which the digestive diverticulum of molluscan bivalves is dissected and processed, is a more appropriate sample preparation method for this food type. Although, there is still potential for molluscan bivalves to become externally contaminated either through contact with other contaminated ingredients, catering surfaces, fomites or through infected food handlers during preparation (Smith *et al.,* 2012). With these contamination sources less likely to occur, this would not justify changing to this method of testing for this particular food group, however the surface wash method is appropriate and applicable to ready to eat foods, for which there is currently a testing gap.

Furthermore modifications to the preparation of certain foods types may be required prior to virus capture, in order to improve the sensitivity of detection by PCR. Some modifications already exist to improve the detection of bacterial targets in the Food, Water and Environment Laboratory Network. Specifically, the addition of skimmed milk powder to cocoa and chocolate based products is conducted to reduce bactericidal properties for the detection of *Salmonella.* Potassium sulphite is also added to onion and garlic based products to reduce bactericidal properties for the detection of *Salmonella.*

A limitation of the capture technique is that due to norovirus strain variability, re-validation of the capture method for new emerging strains or strains not validated in this thesis is required. In this thesis six norovirus strains were validated, however strain variability to bind with HBGAs should be considered in future work, as not all noroviruses interact with HBGAs (Harrington et al., 2002; Huang et al., 2005). Some strains have demonstrated a wider range of HBGA binding patterns than others; therefore the capture technique may be biased towards capturing those norovirus strains which exhibit the greatest number of HGBA binding patterns. However it was identified that norovirus bound non-specifically to the magnetic beads and EDC linker molecule, which may capture those norovirus strains that exhibit no specific binding interactions with HBGAs. Although the capture technique may be less sensitive for those strains that only bind non-specifically, due to competition for binding space on the magnetic beads, linker or PGM molecule.

Despite the many advantages of the sensitivity of PCR for the detection of viruses, the limitation of this technique is that it is designed to detect viral nucleic acid and is unable to differentiate between infectious and non-infectious virus. Although research groups are working towards the development of a cell culture model for norovirus (Ettayebi *et al.,* 2016) without a cell culture system, it is not possible to determine the proportion of infectious norovirus captured from foods. Therefore, we can only report that the foods

were positive for norovirus RNA, highlighting the risk of infection involved in the consumption of that particular food. No further assessment can be made with any great certainty regarding the infectious dose within contaminated food. Interpretation of what proportion of positive PCR signals are infectious is impossible in the absence of a cell culture model and norovirus PCR signals may be generated from a mixture of infectious virus particles and interfering defective virus particles (Knight *et al.,* 2013). The bead capture technique in this thesis allows the detection of norovirus RNA associated with intact virus capsids, therefore eliminating the possibility of detecting free RNA or damaged virus particles; although it is not possible to determine what proportion of those intact virus capsids are infectious.

Alternative molecular approaches such as use of photoactivatable dyes; which fluoresce when excited at the appropriate wavelengths to assess the infectivity of norovirus, are being developed and may help to differentiate between PCR signals generated by infectious and non-infectious material. Human enteroids as a cell culture model are also under development (Ettayebi *et al.,* 2016). These could be used in the recovery of viruses from foods and could be used to reduce over estimation of norovirus recovered from foods by RTqPCR (Randazzo *et al.,* 2016). An assessment of these methods in light of the full protocol could be conducted in future work. Until then questions around infectivity still remain.

Quantification is important in assessing the sensitivity of a detection method, and this is particularly important in the context of food associated viruses, where low level and uneven contamination may be present. The quantification standards used throughout this thesis were dsDNA plasmids. From this curve the results were calculated from plotting the Cq against the $\log_{10}$ number of norovirus cDNA copies. It is acknowledged that there are limitations to using dsDNA plasmid standards to quantify target norovirus in specimens. As

dsDNA standards are subjected to the PCR step only, and do not reflect the genomic structure of the target organism. However, in a study conducted by Costafreda *et al.* (2006), the use of RNA standards compared to dsDNA standards for the purpose of quantification did not result in significant differences. Alternative standard control materials are available that could also be used once validated on the full protocol. These include LENTICULES^{TM} (Hartnell *et al.*, 2012) or standard controls generated by The National Institute for Biological Standards and Control (NIBSC) (Fryer *et al.,* 2008).

Overall the protocol developed in this thesis was validated to screen RTE foods in a high throughput manner in official control laboratories for the detection of norovirus. Although the protocol seems highly specific to norovirus, it was designed with the view to expand the application of it to Hepatitis A virus and Hepatitis E virus. In Chapter 3 non-specific capture of norovirus by PGM magnetic beads was evident and therefore capture of other viruses non-specifically is possible. After virus capture and nucleic acid extraction, cDNA generated using random primers can be applied to PCR assays for the detection of Hepatitis A virus and Hepatitis E virus using primers specifically designed for these targets. ISO 15216 (2013) proposes a PCR assay for the detection of Hepatitis A, therefore future work could include validation of this PCR assay, alongside the PGM capture method and automated nucleic acid extraction described in this thesis, to expand the application of this method to other food borne viruses. The PGM capture and automated nucleic acid extraction method has been applied alongside PCR assays developed by the Blood Borne Virus Unit at Public Health England, to successfully detect Hepatitis A RNA from sewage samples and Hepatitis E RNA from faecal samples.

For all future work mengovirus would be a suitable internal process control as it is structurally similar (a non-enveloped virus) and has a similar genomic structure to norovirus, hepatitis A virus and hepatitis E virus, therefore it can be extracted in the same

way and requires reverse transcription before amplification by PCR. Throughout this thesis the mengovirus strain used was MC_0 a genetically modified strain with a deleted polyC tract. It may not be a suitable internal process control to use in some laboratories as use of genetically modified organisms may not be permitted for health and safety reasons by some laboratories. Although it is non-pathogenic to humans, the use of any genetically modified material requires prior approval from the genetically modified material committee. However as an alternative internal control MS2 could be used, as it is not a genetically modified organisms. It is a suitable internal control as it is a bacteriophage which infects bacterium *Escherichia* coli, commonly found in the gastrointestinal tract, which can also be infected by norovirus, hepatitis A virus and hepatitis E virus.

Mengovirus was used in this protocol in two ways; firstly as a an external extraction control to monitor inter-run variation and secondly as an internal control spike to measure sample associated inhibition, as it is expected to vary from food sample to food sample due to the complex composition of different food types. The mengovirus external extraction control was produced and comprised of a batch of 30 aliquots which were tested independently across 30 runs. The mean Cq value and standard deviations were established against subsequent extractions of the batch, which were tested and validated. Extraction performance in all runs was deemed as acceptable as Cq values of the external extraction control fell within ±3 standard deviations (Appendix A). Monitoring the mengovirus internal control spike was required to monitor inhibitory effects and to identify false negatives. Although the internal control Cq value was detected in norovirus negative samples (Appendix U and V), in future work all internal control Cq values should be checked to see whether they fall within an acceptable validated range of the equivalent internal control spike in no target control samples (such as PBS or water); which represents a sample type with no or little inhibition. If Cq values from negative food samples then deviate from the

acceptable range this is likely to be a marker of inhibition, which may lead to a false negative result. These samples would require further investigation. These food samples should be re-extracted and a one in ten, one in one hundred and one in one thousand preparation of the food sample should be extracted to dilute out any food associated inhibitors, and the RT-PCR for norovirus and mengovirus should be repeated. There is a risk that in low level norovirus contamination, dilution of the sample may result in dilution of norovirus contamination to a level below the sensitivity of the assay, resulting in a false negative result, in this case a half $\log_{10}$ dilution of the food sample could be tested to prevent diluting out low level norovirus contamination to below the level of sensitivity of the assay. If a half $\log_{10}$ dilution is not enough to dilute out the inhibitors, this would be accessed by the Cq value of the internal control in relation to the internal control Cq value in the no target control.

Faecal specimens used in hand contamination experiments were chosen to represent true hand contamination, and are more readily available specimens in comparison to cell culture material, as a cell culture model is not routinely applicable for norovirus yet. The two inoculums used in hand contamination both contained high viral loads, as quantitative data on norovirus transfer was not available from the literature, to help guide the inoculum to use. Attempts were made to adapt the high viral load identified in the faecal specimens for hand contamination simulations, by distributing a small volume onto the non-dominant hand of food handler volunteers. One question it has not been possible to address fully in this study was whether food inoculation experiments with decreasing viral loads, result in an increase or decrease in the amount of norovirus captured from both food and food handler's hands. This can be achieved in future work by inoculating a range of viral loads of norovirus on food handler's hands, and conducting a statistically significant number of food handler contamination experiments. Presumably lower titres of norovirus used in hand

contamination experiments would result in low titres transferred at each stage of food preparation. To answer this research question, dilutions of the inoculum would need to be conducted to simulate real scenarios, to see how lower viral loads transfer during food preparation, with consideration that transfer efficiency can vary depending on the pressure applied (Ansari *et al.,* 1988), contact time, the texture of a contact surface (D'Souza *et al.,* 2006, Escudero *et al.,* 2012) and whether the contact surface is wet or dry (Sharps *et al.,* 2012). Recovery efficiency could also be calculated in future experiments by contaminating the hand and then conducting a wash to elute virus immediately off without transferring to another hand, to establish a baseline of norovirus recovery from gloved hands, to be used in calculating transfer efficiency.

Although bare hand contact has been identified as a risk factor strongly linked to virus transmission (Todd *et al.*, 2007), it was not possible in the thesis, to intentionally contaminate the bare hands of food handler volunteers with known norovirus positive faecal specimens, putting them at an unnecessary risk of acquiring infection. It is possible with ethics approval that the detection of norovirus from bare hand washes could be conducted, and norovirus could be removed using the American Society for Testing and Materials standard test method. In this method the hand is placed inside a bag containing 75 mL stripping solution and massaged for 60 seconds to ensure all hand surfaces are covered with solution (ASTM, 1994). The virus capture, concentration and molecular detection method used in chapter 3, could then be applied to the 75ml hand wash solutions stated in this testing standard. In order to allow for the variation in human behaviour and identify the viral loads transferred by the methods proposed in this study, a statistically significant number of replicate experiments would need to be conducted, using a wide range of food hander volunteers, and not laboratory staff exclusively, as it may be identified that laboratory staff demonstrate a higher level of dexterity, than members of

the public. Alternatively a surrogate for norovirus such as a bacteriophage (MS2) could be used to contaminate bare hands of food handlers, as bacteriophage are non-infectious. Mock up kitchens could be hired to better represent food preparation environments in the home and the food handler protocol could be used to identify the role of food handler transmission in the home, as very little information about food handler contamination when preparing food for family members or guests in the home environment exists. Few studies have analysed domestic food handling behaviours, perhaps because people perceive their homes as being a less likely source of foodborne disease as a consequence of poor hand hygiene. Failure to acknowledge poor food-handling practices in the home and to make changes to food-handling behaviour may be the reason why it was reported in one study that foodborne disease arising from foods consumed in the home is three times more frequent than that arising from foods consumed in a cafeteria (Borneff, 1989). Further research in domestic environments may provide useful insight into food handler contamination. Recruiting a wider range of Food Handler volunteers may also reduce human factors such as repeating experiments on subsequent days, which may have led to cognitive manual handling behaviours, and altered the frequency in which food handlers touched environmental surfaces.

In the food handler experiments designed in this thesis the separation of the dominant and non-dominant hand can be considered but not fully evaluated due to the lack of a large number of replicates. Hands frequently come into contact with environmental surfaces, where they may acquire or deposit micro-organisms. Some studies have investigated micro-organism distribution on hands after completing a task. In a study conducted by Casanova *et al.* (2008) it was identified in 90% of the 10 volunteers, that the dominant hand was used more in the removal of contaminated PPE, therefore more virus was

deposited on it in comparison to the non–dominant hand. This could be explored using the experimental design of the simulation experiments in the future.

5. Conclusion

The specific aims of this PhD thesis were to develop, optimise and validate a broadly reactive virus capture and concentration method to recover norovirus from a range of complex ready-to-eat food matrices.

In Chapter 3 the thesis demonstrates that surface washing provided the best recovery of norovirus from ready-to-eat foods. This was enhanced by capture using PGM conjugated to magnetic beads, added to a sample volume of 50ml incubated at pH 3.5, 4^{o}C for 60 minutes. This methodology led to a sensitive method of capturing norovirus from a range of ready to eat food matrices. From artificial contamination with norovirus GII-5 the sensitivity of the detection method was 8.5 x 10^1 cDNA copies per 25g of strawberries, 4.3 x 10^1 cDNA copies per 25g of lettuce and 1.2 x 10^2 cDNA copies per 25g of ham. The sensitivity of norovirus detected differed depending on the genotype used to artificially contaminate ham. The sensitivity of norovirus GII-4 was 8.0 x 10^1 cDNA copies per 25g of ham, 1.1 x 10^2 cDNA copies of norovirus GI-7 per 25g of ham was detected and 3.5 x 10^2 cDNA copies of norovirus GII-6 per 25g of ham was detected. A sensitive detection method was addressed through the incorporation of a broadly reactive magnetic bead virus capture technique adapted from Tian *et al.,* (2010), alongside a highly sensitive and automated extraction method using the QIAgen QIAsymphony™ platform. Two well established PCR assays were evaluated to improve detection and allow selection of one of these, Kageyama *et al.* (2003), in the final protocol. This resulted in a rapid and highly sensitive methodology, which

incorporates automation, to provide a fit for purpose testing approach for use in an official control laboratory setting.

In Chapter 4 a protocol was devised for simulating food preparation. This protocol was designed considering how to introduce artificial inoculation to best simulate hand contamination using detection methods established in Chapter 3, to allow quantitative detection and monitoring of virus transmission. The data collected was then used to establish the level of norovirus recovered through three tasks of sandwich preparation. From six replicate norovirus GI simulation experiments an average of 7.8 (SD ± 0.1) $\log_{10}$ cDNA copies per glove was inoculated, and an average of 5.9 (SD ± 0.8) $\log_{10}$ cDNA copies of norovirus GII was transferred to 25g of sandwich via two food handlers during the sandwich making process. For norovirus GII simulation experiments an average of 5.9 (SD ± 0.1) $\log_{10}$ cDNA copies per glove was inoculated, and an average of 3.2 (SD ± 0.8) $\log_{10}$ cDNA copies of norovirus GI was transferred to 25g of sandwich via two food handlers during the sandwich making process. Throughout the six replicate simulation experiments the percentage of norovirus GI inoculum recovered from the food, food handler's hands and the environment ranged from 1% to 23% and for norovirus GII ranged from 1% to 32%.

Characterisation methods identified norovirus from food, food handler hands and environmental samples were the same genotype. However application of these methods to the low viral loads indicated that optimisation is required should these become widely used, for example in foodborne outbreak settings. Overall the protocol could be effective for risk assessment models to demonstrate the transfer of norovirus during preparation of foods in catering premises through poor hand hygiene. Further work applying these protocols to quantify the transfer from contaminated hands using a range of viral loads will be useful in determining risk more accurately.

This thesis developed, optimised and validated a method that improved sample throughput and enabled low level virus detection from RTE foods. This method was demonstrated through simulation experiments, which showed that norovirus transfers between food, food handlers and the environment, and that it can be detected at different stages throughout a multistage food preparation process.

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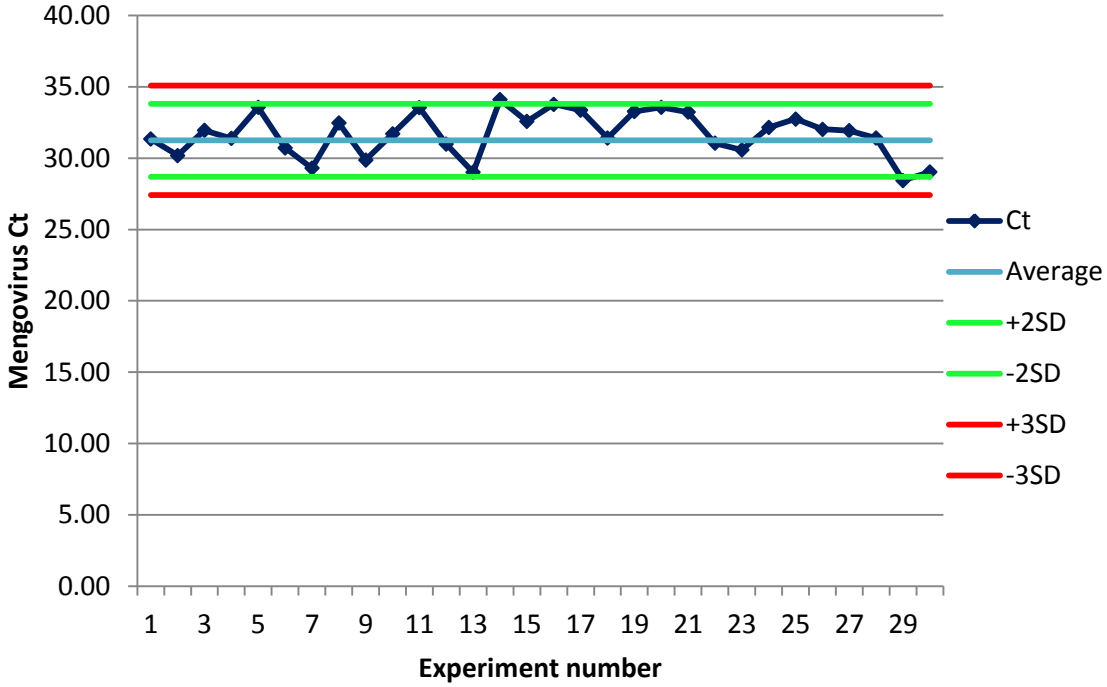
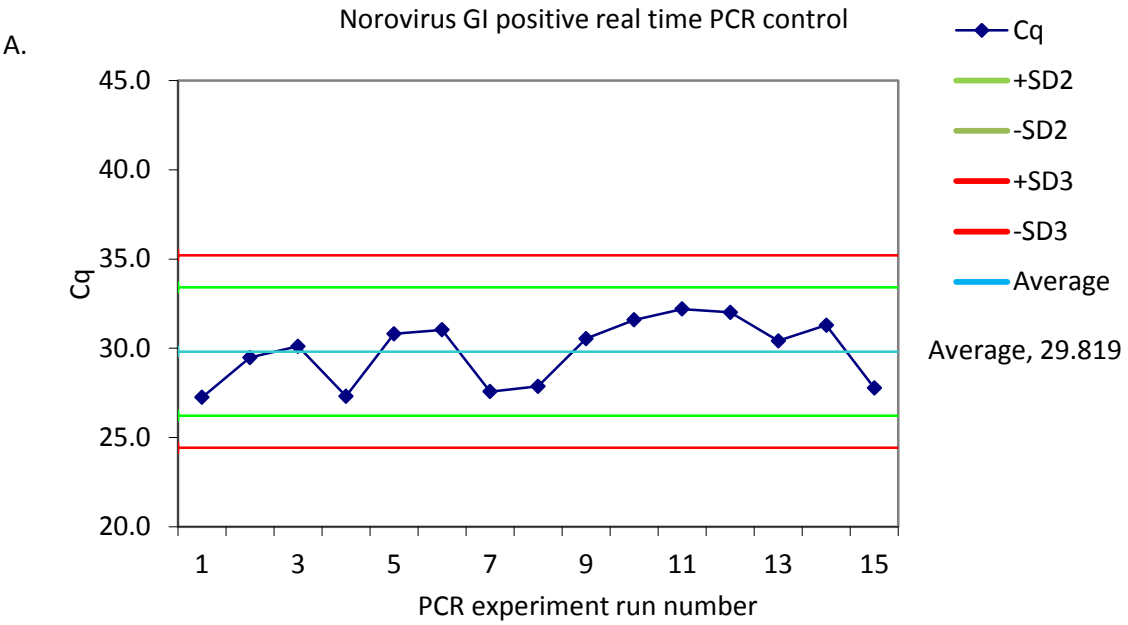
APPENDIX ***A: Quality control for the Mengovirus extraction control Ct values from 3 Kageyama assay real time RTPCR experiments (dark blue line=Cq of mengovirus per experiment, light blue line=average, green line= 2 standard deviations, red line = 3 standard deviations away from the average.***

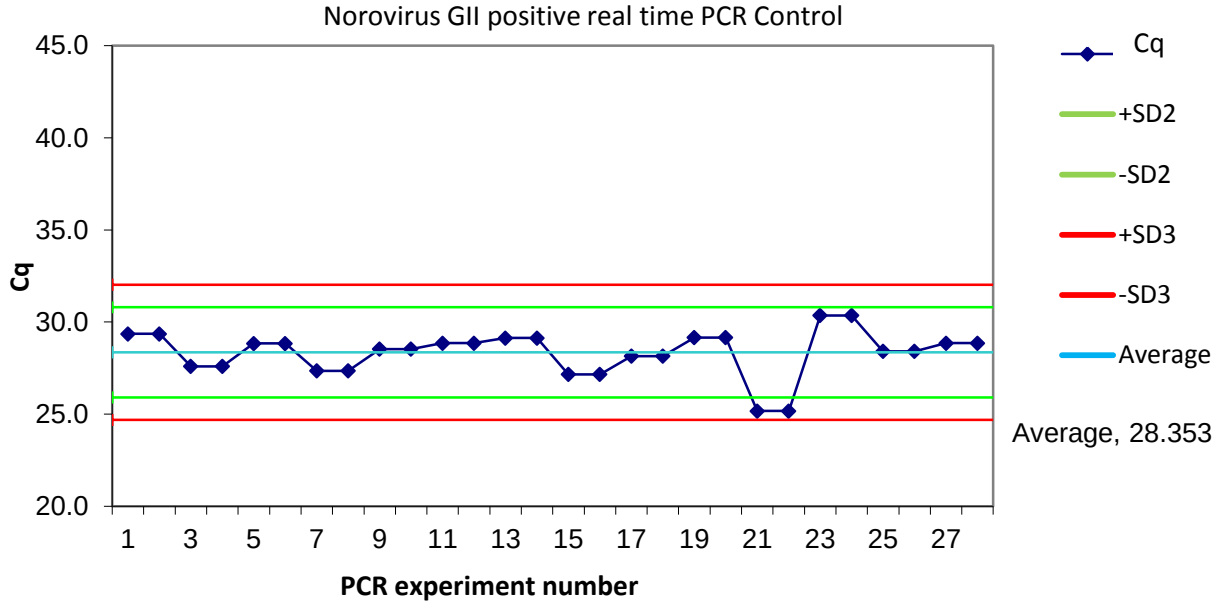


APPENDIX ***B: Quality control for norovirus cDNA controls Cq values from Kageyama assay real time RTPCR experiments (dark blue line=Cq of norovirus per experiment, light blue line=average, green line= 2 standard deviations, red line = 3 standard deviations away from the average. A: 5µl of norovirus GI cDNA control added to PCR mix B: 5µl of norovirus GII cDNA control added to PCR mix***

A.

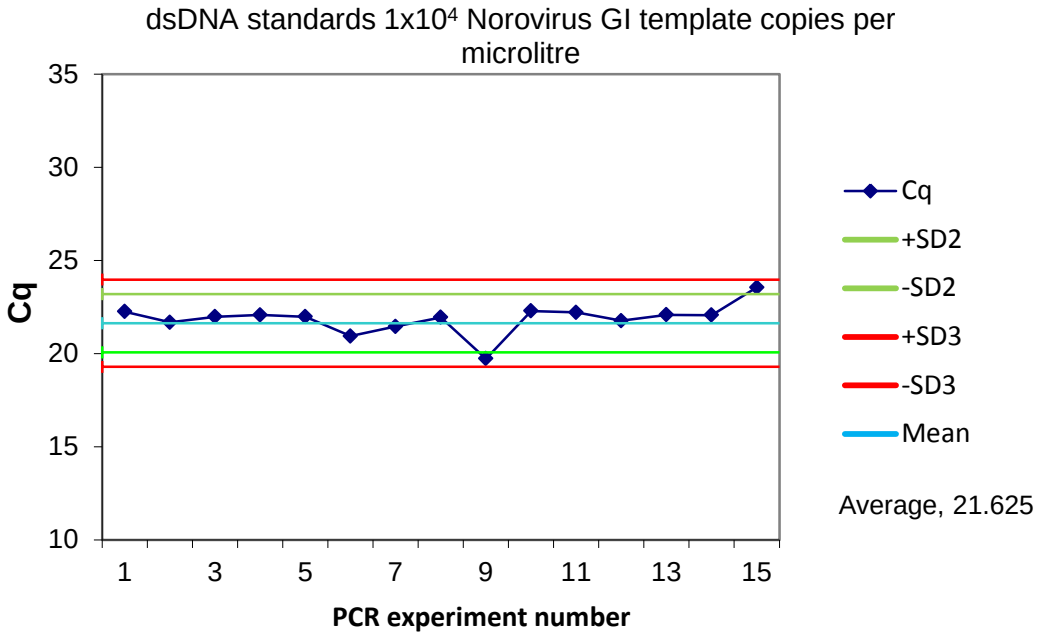


B

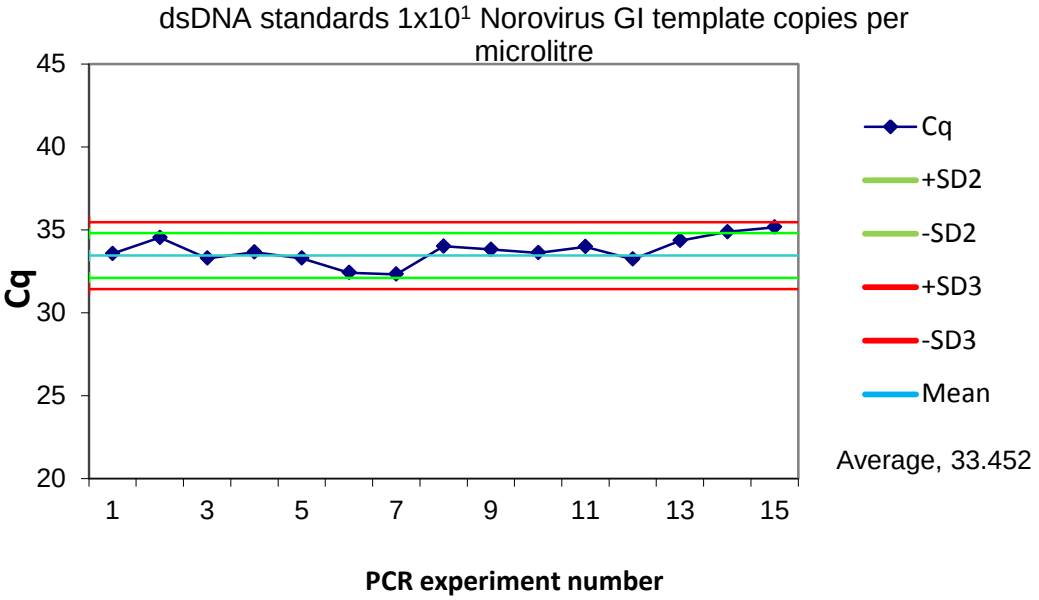


APPENDIX C: Quality control for norovirus dsDNA controls Cq values from 15 Kageyama assay real time RTPCR experiments (dark blue line=Cq of norovirus per experiment, light blue line=average, green line= 2 standard deviations, red line = 3 standard deviations away from the average. A: 5µl of norovirus GI dsDNA control added to PCR mix at 10^4 dsDNA copies/µl B: 5µl of norovirus GI dsDNA control added to PCR mix at 10^1 dsDNA copies/µl

A.

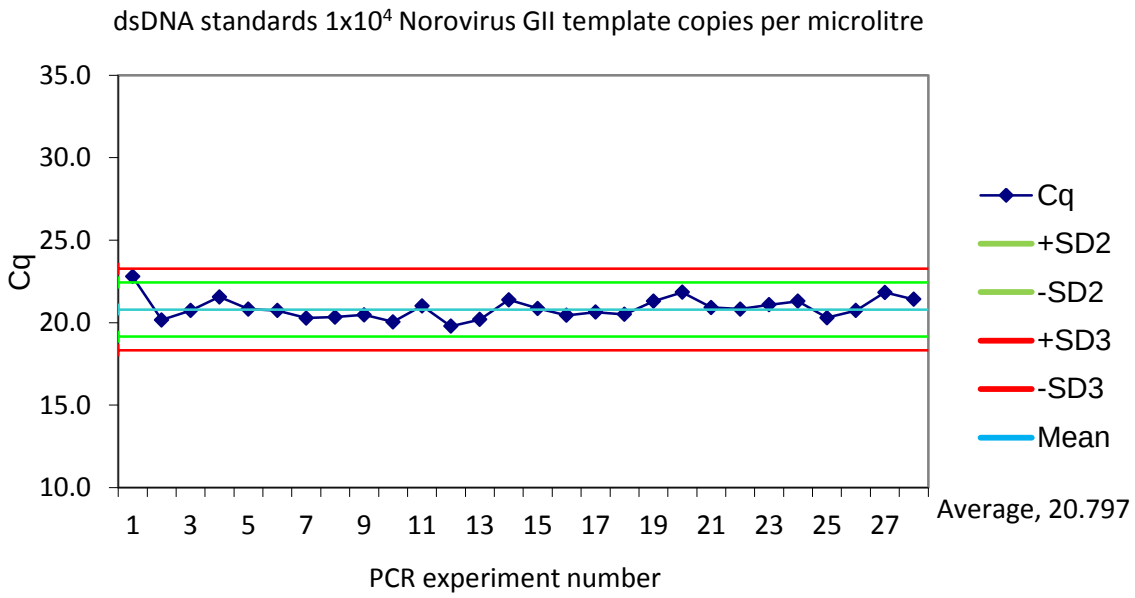


B.

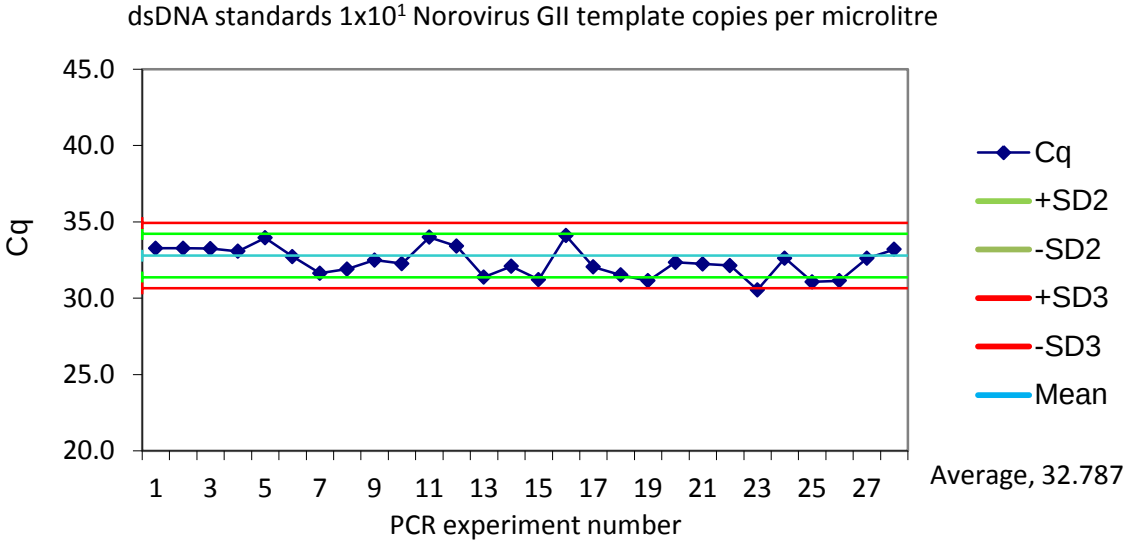


APPENDIX D: Quality control for norovirus dsDNA controls Cq values from 30 Kageyama assay real time RTPCR experiments (dark blue line=Cq of norovirus per experiment, light blue line=average, green line= 2 standard deviations, red line = 3 standard deviations away from the average. A: 5µl of norovirus GII dsDNA control added to PCR mix at 10^4 dsDNA copies/µl B: 5µl of norovirus GII dsDNA control added to PCR mix at 10^1 dsDNA copies/µl

A.



B.



APPENDIX E: Cq value of three biological repeats of norovirus (GII) captured from 50ml of PBS using 0.15mg/ml, 0.38mg/ml and 0.75mg/ml of PGM on magnetic beads the average Ct, Standard deviation and ±95%CI

Norovirus serial dilution	Neat	Neat	Neat		1:10	1:10	1:10		1:100	1:100	1:100
PGM concentration	0.15mg/ml	0.38mg/ml	0.75mg/ml l		0.15mg/ml	0.38mg/ml	0.75mg/ml		0.15mg/ml	0.38mg/ml	0.75mg/ml
Reading 1	19.3	20.7	21.7		25.0	24.1	24.0		29.1	28.9	27.7
Reading 2	20.6	21.4	21.4		25.4	25.2	25.3		29.6	30.3	29.6
Reading 3	22.2	21.3	21.1		25.0	25.3	24.8		28.1	29.1	28.5
Mean	20.7	21.1	21.4		25.1	24.9	24.7		28.9	29.5	28.6
Standard deviation	1.5	0.4	0.3		0.3	0.7	0.7		0.8	0.8	1.0
confidence (95%)	3.6	1.0	0.8		0.6	1.7	1.6		1.9	1.9	2.4
Max Ct (±95% CI)	24.3	22.1	22.2		25.8	26.6	26.3		30.8	31.4	31.0
Min (±95% CI)	17.1	20.2	20.6		24.5	23.1	23.1		27.0	27.6	26.2

APPENDIX F: Average Cq value and standard deviation of norovirus GII inoculum across a ten-fold serial dilution (Neat to 10-4) artificially contaminate onto the surface of RTE food, surface washed in three different wash volumes (5ml, 50ml & 100ml) of PBS (pH7), and captured using PGM coated magnetic beads (100µl) from a stock concentration of 7.5mg/ ml of PGM, in triplicate ±95%CI

	5ml RTE food surface wash		50ml RTE food surface wash		100ml RTE food surface wash	
Raspberries						
	average	±95%CI	average	±95%CI	average	±95%CI
10% Suspension	23.2	0.6	20.4	0.6	21.6	0.8
1:100	31.1	0.8	24.9	0.4	25.7	0.8
1:1,000	33.0	0.4	27.2	0.6	31.4	0.3
1:10,000	nvd	nvd	37.0	0.3	34.8	0.6
1:100,000	nvd	nvd	36.2	1.8	nvd	nvd
Lettuce						
	average	±95%CI	average	±95%CI	average	±95%CI
10% Suspension	24.6	1.0	22.4	1.2	22.8	1.7
1:100	34.2	4.9	27.2	1.0	27.2	0.9
1:1,000	33.6	1.0	32.2	0.9	30.1	0.9
1:10,000	38.8	0.5	35.6	1.3	35.3	1.0
1:100,000	nvd	nvd	35.9	1.0	38.7	1.0
Ham						
	average	±95%CI	average	±95%CI	average	±95%CI
10% Suspension	24.0	1.0	18.2	0.4	18.5	0.2
1:100	25.2	1.3	22.2	0.3	22.9	0.3
1:1,000	27.5	0.3	26.3	0.3	26.4	0.3
1:10,000	31.5	0.1	30.4	0.3	30.7	0.0
1:100,000	34.1	0.4	33.7	0.5	34.5	0.2
	38.1	0.2	36.9	0.4	37.1	0.4

APPENDIX G: Average Cq values of norovirus GI and GII recovery in different pH buffers (pH 3.5-10.0) conducted in duplicate across two runs

	Norovirus GI			Norovirus GII		
	pH 3.5	pH 7	pH 10.0	pH 3.5	pH 7	pH 10.0
Reading 1	33.22	35.01	35.52	24.96	27.41	25.24
Reading 2	33.57	34.71	35.18	25.00	26.82	24.52
Reading 1	32.89	36.92	39.37	25.68	27.02	27.04
Reading 2	31.19	34.29	38.94	25.28	28.31	26.78
mean	32.72	35.23	37.26	25.23	27.39	25.89
SD.	1.05	1.16	2.21	0.33	0.66	1.21
median	33.05	34.86	37.23	25.14	27.22	26.01
q1	32.47	34.61	35.44	24.99	26.97	25.06
min	31.19	34.29	35.18	24.96	26.82	24.52
max	33.57	36.92	39.37	25.68	28.31	27.04
q3	33.30	35.49	39.05	25.38	27.64	26.84
q1-minimum	1.27	0.32	0.25	0.03	0.15	0.54
q1	32.47	34.61	35.44	24.99	26.97	25.06
median-q1	0.59	0.26	1.79	0.15	0.25	0.95
q3-median	0.25	0.63	1.82	0.24	0.42	0.83
maximum-q3	0.27	1.43	0.33	0.30	0.67	0.19

APPENDIX H: Cq values norovirus GI inoculum contaminated on berries washed in PBS and pH adjusted (pH 3.5 or pH 10.0) compared to berries washed in PBS non pH adjusted (pH 6.5-6.8), carried out in triplicate across two inter-repeats (raw data)

	Raspberries pH 3.5	Raspberries pH 6.8	Raspberries pH 10.0	Strawberries pH 3.5	Strawberries pH 7.2	Strawberries pH 10.0
Reading 1	32.11	34.81	34.32	30.48	35.48	36.23
Reading 2	30.89	32.96	34.95	29.91	37.08	36.59
Reading 3	31.72	33.86	35.15	30.64	36.7	34.17
Reading 1	31.5	33.56	34.57	31.31	37.78	35.12
Reading 2	30.28	34.31	37.02	31.11	35.71	37.89
Reading 3	31.76	33.36	33.18	31.37	37.05	37.99
mean	31.38	33.81	34.87	30.8	36.63	36.33
s.d.	0.67	0.67	1.26	0.57	0.88	1.51
median	31.61	33.71	34.76	30.88	36.87	36.41
q1	31.04	33.41	34.38	30.52	35.96	35.4
min	30.28	32.96	33.18	29.91	35.48	34.17
max	32.11	34.81	37.02	31.37	37.78	37.99
q3	31.75	34.2	35.1	31.26	37.08	37.57
q1-minimum	0.76	0.45	1.2	0.61	0.48	1.23
q1	31.04	33.41	34.38	30.52	35.96	35.4
median-q1	0.56	0.3	0.38	0.36	0.91	1.01
q3-median	0.14	0.49	0.34	0.74	0.2	1.16
maximum-q3	0.36	0.61	1.92	0.1	0.7	0.42

APPENDIX I: Cq values of norovirus GII inoculum contaminated on berries washed in PBS and pH adjusted (pH3.5 or pH 10) compared to natural pH of berries washed in PBS (pH 6.5-6.8)) carried out in triplicate across two inter-repeats (raw data).

	Raspberries pH 3.5	Raspberries pH 6.9	Raspberries pH 10.0	Strawberries pH 3.5	Strawberries pH 7.1	Strawberries pH 10.0
Reading 1	21.76	30.31	27.53	27.03	27.85	27.54
Reading 2	26.06	33.7	27.04	26.16	28.68	27.75
Reading 3	22.16	29.95	27.75	26.19	28.68	24.3
Reading 4	24.24	30.72	27.24	26.56	28.64	33.09
Reading 5	30.46	30.62	28.21	24.89	28.4	31.91
Reading 6	25.17	30.51	26.65	28.23	29.05	27.71
mean	24.98	30.97	27.4	26.51	28.55	28.72
SD.	3.16	1.37	0.55	1.1	0.4	3.23
median	24.71	30.56	27.14	26.38	28.66	27.73
q1	22.68	30.36	27.09	26.17	28.46	27.59
min	21.76	29.95	26.65	24.89	27.85	24.3
max	30.46	33.7	28.21	28.23	29.05	33.09
q3	25.84	30.69	27.69	26.91	28.68	30.87
q1-minimum	0.91	0.41	0.43	1.28	0.62	3.29
q1	22.68	30.36	27.09	26.17	28.46	27.59
median-q1	2.03	0.21	0.05	0.21	0.2	0.15
q3-median	1.13	0.13	0.55	0.54	0.02	3.14
maximum-q3	4.62	3.01	0.52	1.32	0.37	2.22

APPENDIX J: Average Cq of norovirus GII-4 Inoculum captured from 50ml of PBS (pH3.5) incubated at three different temperatures (4^0c, 37^0c, ambient temperature) recorded at 4 different time points (30mins, 60mins, 120mins and overnight) in duplicate across three inter-repeats including upper and lower quartiles(raw data).

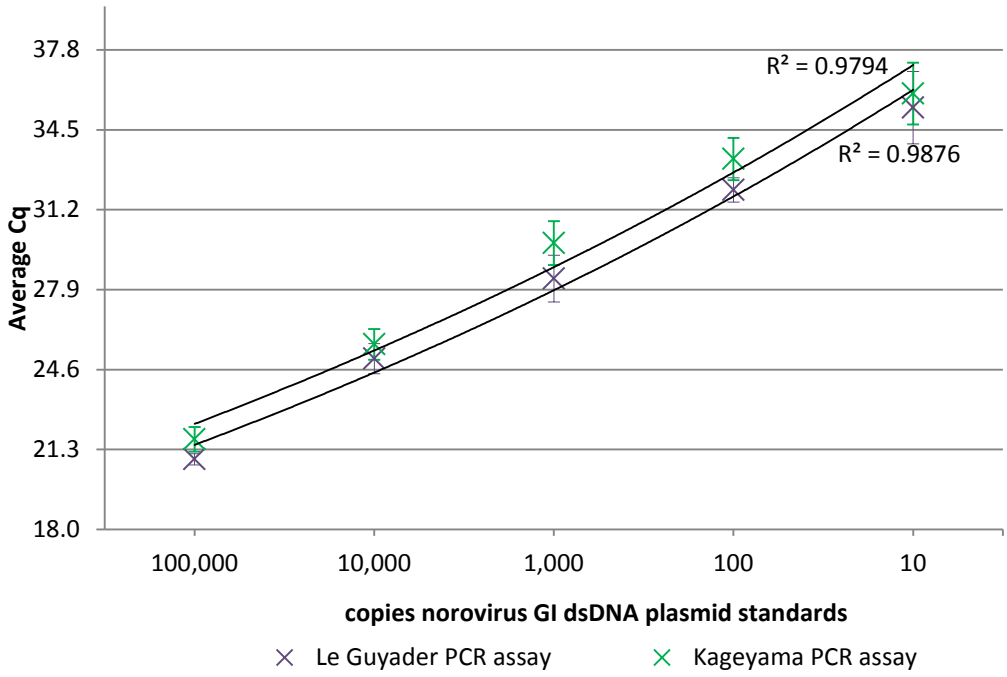
	inoculum	30mins	60mins	120mins	overnight	30mins	60mins	120mins	overnight	30mins	60mins	120mins	overnight
		37°c	37°c	37°c	37°c	4°c	4°c	4°c	4°c	AT	AT	AT	AT
reading 1	27.46	28.37	28.09	29.48	29.4	28.13	27.4	28.19	28.93	28.34	27.19	29.17	29.22
reading 2	28.05	28.34	28.07	29.26	30.1	28.58	27.16	27.63	28.34	28.94	27.42	28.21	28.93
reading 3	28.51	29.92	29.04	30.09	29.97	29.05	27.76	28.5	28.43	28.79	26.85	29.16	28.5
reading 4	27.95	29.3	28.84	29.58	31.25	29.13	28.37	28.51	28.01	28.75	28.96	29.38	28.49
reading 5	28.23	29.06	28.57	29.1	29.33	27.53	26.87	27.3	28.19	27.54	27.82	27.4	29.3
reading 6	28.08	28.73	28.62	28.68	29.25	28.04	27.4	27.76	27.74	27.95	28.37	27.96	27.71
mean	28.05	28.95	28.54	29.37	29.88	28.41	27.49	27.98	28.27	28.38	27.77	28.55	28.69
SD.	0.35	0.61	0.39	0.48	0.76	0.62	0.52	0.5	0.41	0.55	0.79	0.8	0.59
median	28.06	28.89	28.59	29.37	29.68	28.35	27.4	27.98	28.27	28.55	27.62	28.68	28.72
q1	27.98	28.46	28.21	29.14	29.35	28.06	27.22	27.66	28.05	28.05	27.25	28.02	28.5
min	27.46	28.34	28.07	28.68	29.25	27.53	26.87	27.3	27.74	27.54	26.85	27.4	27.71
max	28.51	29.92	29.04	30.09	31.25	29.13	28.37	28.51	28.93	28.94	28.96	29.38	29.3
q3	28.3	29.46	28.89	29.71	30.38	29.07	27.92	28.51	28.56	28.82	28.52	29.22	29.24
q1-minimum	0.52	0.12	0.14	0.46	0.1	0.53	0.36	0.36	0.32	0.5	0.4	0.62	0.79
q1	27.98	28.46	28.21	29.14	29.35	28.06	27.22	27.66	28.05	28.05	27.25	28.02	28.5
median-q1	0.09	0.43	0.38	0.24	0.34	0.29	0.18	0.32	0.21	0.5	0.37	0.66	0.22
q3-median	0.24	0.56	0.29	0.34	0.7	0.71	0.52	0.53	0.29	0.28	0.9	0.54	0.52
maximum-q3	0.21	0.47	0.15	0.38	0.87	0.06	0.46	0.01	0.37	0.11	0.44	0.16	0.06

APPENDIX K: average Cq values of norovirus GII-4 inoculum captured from PBS, strawberries, lettuce and ham (pH3.5) incubated at 4℃ or ambient temperature for 60 minutes carried out in duplicate including upper and lower quartiles(raw data)

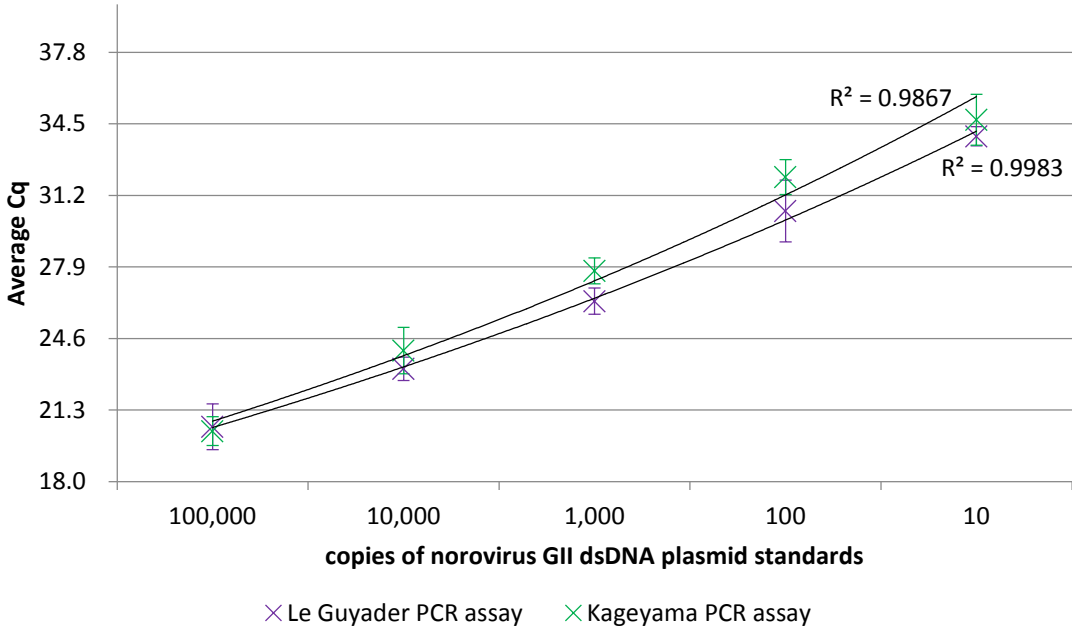
	inoculum	50ml bead capture control	strawberries	lettuce	ham	strawberries	lettuce	ham
Reading 1	2.90E+06	1.09E+06	1.31E+06	5.70E+05	9.60E+05	2.12E+05	3.51E+05	3.00E+05
Reading 2	2.67E+06	9.04E+05	9.08E+01	3.88E+05	1.08E+06	2.88E+05	3.47E+05	6.60E+05
Reading 3	2.75E+06	7.55E+05	2.20E+05	3.21E+05	2.04E+05	3.90E+02	3.44E+05	1.50E+05
Reading 4	2.75E+06	1.05E+06	7.99E+05	5.91E+05	2.52E+05	2.14E+05	2.49E+05	2.10E+05
Average cDNA copies	2.77E+06	9.51E+05	5.83E+05	4.68E+05	6.24E+05	1.79E+05	3.23E+05	3.30E+05
Average $\log_{10}$ cDNA copies	6.44	5.98	5.77	5.67	3.22	5.25	5.51	3.07
Standard deviation of cDNA copies	9.47E+04	1.54E+05	5.91E+05	1.34E+05	4.60E+05	1.24E+05	4.94E+04	2.28E+05

Appendix L: A:Average Cq value of norovirus GI dsDNA standards containing 10 to 100,000 dsDNA copies obtained from the Le Guyader assay (purple) and Kageyama assay (green) PCR assays ±95% CI B:Average Ct of norovirus GII dsDNA standards containing 10 to 100,000 dsDNA copies from the Le Guyader assay (green) and Kageyama assay (purple) PCR assays ±95% CI to demonstrate PCR assay comparability

A.

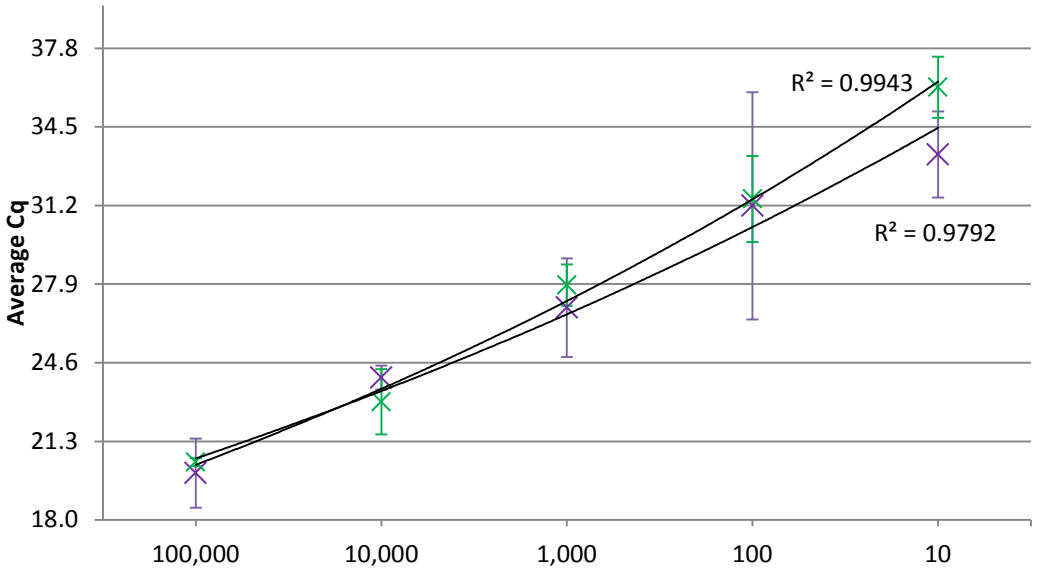


B.

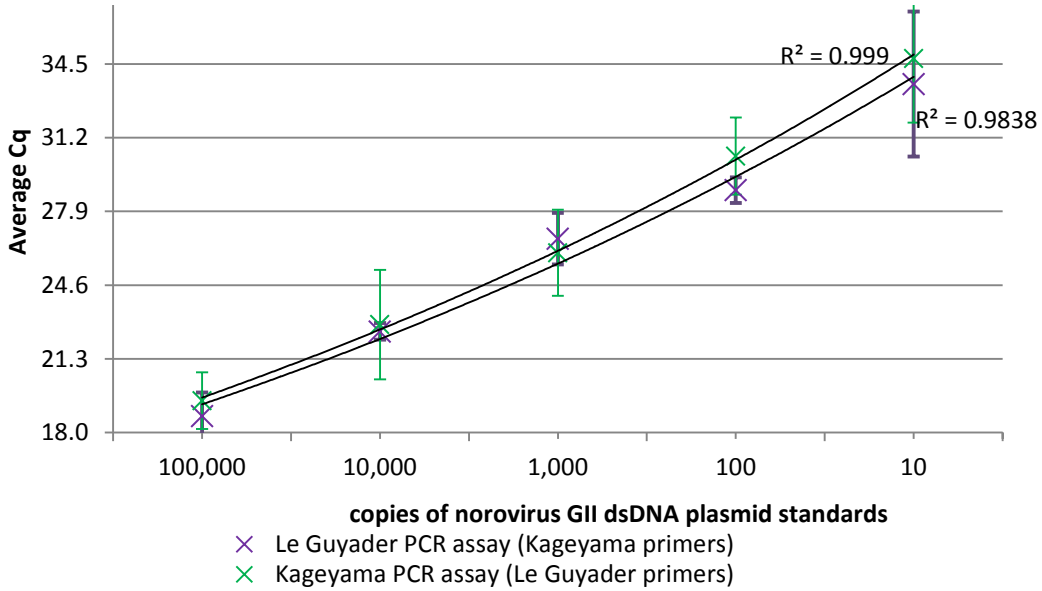


Appendix L: A: Average Cq of norovirus GI dsDNA standards containing 10 to 100,000 dsDNA copies with primer sets exchanged the Le Guyader PCR assay with Kageyama primers (purple) and Kageyama PCR assay with the Le Guyader primers (green) ±95% CI B: Average Cq of norovirus GII dsDNA standards containing 10 to 100,000 dsDNA copies with primer sets exchanged the Le Guyader PCR assay with Kageyama primers (purple) and Kageyama PCR assay with the Le Guyader primers (green) ±95% CI to demonstrate PCR assay comparability

A.



B.



APPENDIX M: A comparison of the sensitivity and quality of total nucleic acid extracts from four automated extraction platforms when processing an inoculum of norovirus GII across a ten-fold dilution series, from four replicates. nvd= no virus detected

Norovirus	Roche MagNA Pure 96™		QIAgen QIAsymphony™		QIAgen QIAxtractor™		Promega Maxwell 16™	
	Average	SD	Average	SD	Average	SD	Average	SD
Neat	15.3	0.2	15.6	0.2	16.1	0.2	21	0.8
10^{-1}	18.5	0.3	19.6	0.4	18.8	0.5	24.8	0.7
10^{-2}	23.1	0.4	23.8	0.3	22.8	0.9	28.8	0.4
10^{-3}	27.5	0.4	27.7	0.4	26.4	0.7	32.8	0.4
10^{-4}	30.8	0.4	31	0.4	30.2	0.4	35.3	0.6
10^{-5}	34	0.4	34.3	0.7	33.5	0.7	35.4	0.1
10^{-6}	37.1	0.7	36.8	0.7	nvd		nvd	
Mengovirus	Roche Mag*NA*P96™		QIAgen QIAsymphony™		QIAgen QIAxtractor™		Promega Maxwell 16™	
	Average	SD	Average	SD	Average	SD	Average	SD
Neat	29.1	0.1	29.4	0.5	30.1	1.7	37.3	nvd
10^{-1}	29.5	0.9	29.6	0.4	29.2	0.1	nvd	nvd
10^{-2}	29.3	0.2	28.8	0.4	31.5	4	nvd	nvd
10^{-3}	29.2	0.2	29.4	0.4	28.8	0.3	37.5	0.8
10^{-4}	29.5	0.2	29.8	0.8	29.4	0.3	37.3	nvd
10^{-5}	29.2	0.3	30.1	0.5	30.9	3.8	36.7	1.2
10^{-6}	29.7	0.5	30.1	0.7	30.4	0.9	38.7	0.4

APPENDIX N: A table presenting Cq values of norovirus GII positive faecal inoculum in ten-fold serial dilutions extracted by Roche MP96™ and QIAgen QIAsymphony™ extraction platforms from four replicates and the average Cq of all four replicates and 95%CI *=average generated from less than four replicates

Norovirus GII ten-fold dilution series	Roche MagNA pure 96™						QIAgen QIAsymphony™					
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	95%CI	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	95%CI
Neat	15.0	15.5	15.3	15.1	15.2	0.3	15.5	15.5	15.6	15.8	15.6	0.2
10-1	18.6	18.5	18.5	18.4	18.5	0.1	19.3	19.4	19.4	19.8	19.5	0.3
10-2	22.8	22.9	23.1	22.9	22.9	0.2	23.3	23.5	23.9	24.1	23.7	0.6
10-3	26.9	26.8	27.5	27.0	27.0	0.5	27.0	27.6	27.5	27.7	27.5	0.5
10-4	30.4	31.0	30.8	30.1	30.6	0.6	30.5	30.9	30.9	31.1	30.8	0.4
10-5	34.3	34.2	34.0	33.8	34.1	0.3	35.3	34.0	34.7	34.5	34.7	0.9
10-6	nvd	36.8	37.1	37.3	37.0*	0.6	nvd	37.3	nvd	36.3	36.8*	6.5
10-7	36.6	nvd	nvd	nvd	36.6*	n/a	nvd	nvd	nvd	nvd	nvd	n/a

APPENDIX O: One-way ANOVA test for Kageyama and Le Guyader assay comparison

A: ANOVA output for norovirus GII- 4 detection

Groups	*Count*	*Sum*	*Average*	*Variance*
Inoculum GII-4 (Kageyama assay)	5	3.38E+08	6.77E+07	1.77E+14
Inoculum GII-4 (Le Guyader assay)	5	2.43E+08	4.85E+07	3.51E+14

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.14E+14	1	9.14E+14	3.456377	0.100064	5.317655
Within Groups	2.11E+15	8	2.64E+14			
Total	3.03E+15	9				

B: ANOVA output for norovirus GII- 5 detection

Groups	*Count*	*Sum*	*Average*	*Variance*
InoculumGII-5 (Kageyama assay)	5	1.47E+08	2.95E+07	1.14E+14
Inoculum GII-5 (Le Guyader assay)	5	2.32E+08	4.63E+07	2.46E+14

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.11E+14	1	7.11E+14	3.951512	0.082047	5.317655
Within Groups	1.44E+15	8	1.80E+14			
Total	2.15E+15	9				

C: ANOVA output for norovirus GII- 6 detection

Groups	*Count*	*Sum*	*Average*	*Variance*
Inoculum GII-6 (Kageyama assay)	5	2.32E+08	4.63E+07	2.46E+14
Inoculum GII-6 (Le Guyader assay)	5	88314650	1.77E+07	2.92E+13

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.05E+15	1	2.05E+15	14.93466	0.004778	5.317655
Within Groups	1.10E+15	8	1.37E+14			
Total	3.15E+15	9				

D: ANOVA output for norovirus GI-7 detection

Groups	*Count*	*Sum*	*Average*	*Variance*
Inoculum GI-7 (Kageyama assay)	5	1.31E+09	2.62E+08	2.12E+16
Inoculum GI-7 (Le Guyader assay)	5	8.41E+08	1.68E+08	7.43E+15

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.22E+16	1	2.22E+16	1.554671	0.247714	5.317655
Within Groups	1.14E+17	8	1.43E+16			
Total	1.37E+17	9				

APPENDIX P: Standard curve data used to determine PCR assay efficiency the Le Guyader and Kageyama assays across 4 norovirus strains and three different food matrices; strawberries, lettuce and ham.

norovirus strain	standard curve information	Le Guyader assay	Kageyama assay
norovirus GII-5 on strawberries	Slope	-3.8	-3.9
	Intercept	34.5	37.0
	R^2	0.997	0.999
	norovirus PCR control	26.36	27.88
	PCR threshold	0.006	0.007
	PCR Efficiency (%)	83%	80%
norovirus GII-5 on lettuce	Slope	-3.4	-3.9
	Intercept	31.589	36.582
	R^2	1.000	0.999
	norovirus PCR control	26.39	27.40
	PCR threshold	0.001	0.004
norovirus GII-5 on ham	Slope	-3.2	-3.4
	Intercept	32.3	36.3
	R^2	1.000	0.997
	norovirus PCR control	26.39	27.40
	PCR threshold	0.001	0.004
	PCR Efficiency (%)	105%	97%
norovirus GII-4 on ham	Slope	-3.3	-4.0
	Intercept	31.5	36.4
	R2	0.999	1.000
	norovirus PCR control	26.48	27.13
	PCR threshold	0.002	0.005
	PCR Efficiency (%)	101%	78%
norovirus GII-6 on ham	Slope	-4.6	-3.6
	Intercept	37.571	32.250
	R2	1.000	1.000
	norovirus PCR control	26.48	27.13
	PCR threshold	0.002	0.005
	PCR Efficiency (%)	65%	90%
norovirus GI-7 on ham	Slope	-3.6	-3.8
	Intercept	35.3	39.0
	R2	0.999	0.999
	norovirus PCR control	29.47	27.41
	PCR threshold	0.005	0.002
	PCR Efficiency (%)	90%	83%

APPENDIX Q: Evaluation of three different methods to inoculate left gloved hand and transfer to Right gloved hand, applying a consistent total volume of 200µl of a 10% faecal suspension (Inoculum) carried out in duplicate and the log cDNA copies/hand recovered

Sample Name	Replicate 1	Replicate 2	Average of 2 replicates
200ul inoculum	6	6	6
50µl inoculum control	6	5.7	5.8
Left Hand 40µlper digit	5.2	5.6	5.4
Right Hand 40µl per digit	4.4	4.6	4.5
Left Hand 200µl in palm	4.2	4.7	4.5
Right Hand 200µl in palm	4.3	4.4	4.4
Left Hand twenty 10µl along fingers	4.7	5.3	5.0
Right Hand twenty 10µl along fingers	3.2	3.5	3.4

APPENDIX R: number of norovirus GI cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 1 & 2.

	Slope	**Intercept**	**R2**	**Amplification factor = 1.93**				
	-3.51462	**35.75144**	**0.99**	**Efficiency = 92.54%**				
GI Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Positve control	inoculum	15	4.60E+07	7.7	inoculum	15	4.34E+07	7.6
	inoculum	15	5.93E+07	7.8	inoculum	15	5.06E+07	7.7
Food Handler 1	Left Hand	18	5.90E+06	6.8	Left Hand	18	7.19E+06	6.9
	Right Hand	22	4.96E+05	5.7	Right Hand	19	3.03E+06	6.5
Food handler 2	Left Hand	24	1.02E+05	5.0	Left Hand	26	3.62E+04	4.6
	Right Hand	27	2.07E+04	4.3	Right Hand	32	5.55E+02	2.7
Food handler 3	Left Hand	35	9.39E+01	2.0	Left Hand	30	2.14E+03	3.3
	Right Hand	33	3.68E+02	2.6	Right Hand	32	7.76E+02	2.9

APPENDIX R: number of norovirus GI cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 3 & 4.

	Slope	**Intercept**	**R2**	**Amplification factor = 2.01**				
	-3.29179	**35.19197**	**0.83**	**Efficiency = 101.27%**				
GI Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Postive control	inoculum	15	7.18E+07	7.9	inoculum	15	7.59E+07	7.9
	inoculum	15	7.76E+07	7.9	inoculum	15	1.03E+08	8.0
Food handler 1	Left Hand	24	1.69E+05	5.2	Left Hand	19	4.37E+06	6.6
	Right Hand	25	9.89E+04	5.0	Right Hand	21	1.30E+06	6.1
Food handler 2	Left Hand	28	7.21E+03	3.9	Left Hand	28	7.21E+03	3.9
	Right Hand	30	2.39E+03	3.4	Right Hand	30	2.39E+03	3.4
Food handler 3	Right Hand	28	8.25E+03	3.9	Left Hand	36	3.13E+01	1.5
	Left Hand	28	1.16E+04	4.1	Right Hand	38	1.19E+01	1.1

APPENDIX R: number of norovirus GI cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 5 & 6. nvd=no virus detected

	Slope	**Intercept**	**R2**	**Amplification factor = 1.91**				
	-3.56	**36.82**	**0.98**	**Efficiency = 90.98%**				
GI Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Positve control	Inoculum	15	7.95E+07	7.9	Inoculum	15	7.48E+07	7.9
	Inoculum	15	7.61E+07	7.9	Inoculum	15	7.40E+07	7.9
Food Handler 1	Left Hand	22	1.00E+06	6.0	Left Hand	19	5.52E+06	6.7
	Right Hand	24	2.12E+05	5.3	Right Hand	19	5.08E+06	6.7
Food handler 2	Left Hand	34	4.05E+02	2.6	Left Hand	32	1.19E+03	3.1
	Right Hand	30	3.78E+03	3.6	Right Hand	30	5.74E+03	3.8
Food handler 3	Left Hand	nvd	nvd	nvd	Left Hand	31	2.48E+03	3.4
	Right Hand	nvd	nvd	nvd	Right Hand	35	1.74E+02	2.2

APPENDIX S: number of norovirus GII cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 1 & 2. nvd=no virus detected

	Slope	**Intercept**	**R2**	**Amplification factor = 2.03**				
	-3.25	**32.08**	**1.00**	**Efficiency = 103.30%**				
GII Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Positive Control	Inoculum	19	7.22E+05	5.9	Inoculum	19	6.01E+05	5.8
	Inoculum	19	7.90E+05	5.9	Inoculum	19	5.68E+05	5.8
Food handler 1	Left Hand	20	1.25E+05	5.1	Left Hand	24	1.37E+04	4.1
	Right Hand	21	1.18E+05	5.1	Right Hand	29	5.10E+02	2.7
Food handler 2	Left Hand	29	5.43E+02	2.7	Left Hand	34	1.94E+01	1.3
	Right Hand	28	8.55E+02	2.9	Right Hand	33	2.84E+01	1.5
Food handler 3	Left Hand	31	1.62E+02	2.2	Left Hand	nvd	nvd	nvd
	Right Hand	33	2.37E+01	1.4	Right Hand	34	1.13E+01	1.1

APPENDIX S: number of norovirus GII cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 3 & 4. nvd=no virus detected

	Slope	**Intercept**	**R2**	**Amplification factor = 1.95**				
	-3.44	**33.39**	**0.99**	**Efficiency = 95.34%**				
GII Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Positive Control	50ml control	19	6.71E+05	5.8	50ml control	19	1.03E+06	6.0
	50ml control	19	8.25E+05	5.9	50ml control	19	1.05E+06	6.0
Food handler 1	Left Hand	28	2.98E+03	3.5	Left Hand	25	1.73E+04	4.2
	Right Hand	32	1.64E+02	2.2	Right Hand	26	7.68E+03	3.9
Food handler 2	Left Hand	37	4.19E+00	0.6	Left Hand	nvd	nvd	nvd
	Right Hand	nvd	5.24E-01	nvd	Right Hand	27	3.49E+03	3.5
Food handler 3	Left Hand	nvd		nvd	Left Hand	38	3.39E+00	0.5
	Right Hand	34	3.75E+01	1.6	Right Hand	31	3.14E+02	2.5

APPENDIX S: number of norovirus GII cDNA copies per µl of extract transferred from food handlers, food and the environment in food handler simulation experiment 5 & 6. nvd=no virus detected

	Slope	**Intercept**	**R2**	**Amplification factor = 1.90**				
	-3.57	**33.50**	**1.00**	**Efficiency = 90.49%**				
GII Norovirus	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT	Sample Name	Cq	cDNA copies per µl of RNA EXTRACT	Log cDNA copies per µl of RNA EXTRACT
Positive Control	Inoculum	19	8.09E+05	5.9	Inoculum	18	1.21E+06	6.1
	Inoculum	19	8.40E+05	5.9	Inoculum	18	1.14E+06	6.1
Food handler 1	Left Hand	26	7.47E+03	3.9	Left Hand	27	3.14E+03	3.5
	Right Hand	28	2.05E+03	3.3	Right Hand	28	1.53E+03	3.2
Food handler 2	Left Hand	nvd		nvd	Left Hand	32	1.32E+02	2.1
	Right Hand	32	1.30E+02	2.1	Right Hand	nvd	nvd	nvd
Food handler 3	Left Hand	32	1.43E+02	2.2	Left Hand	31	3.00E+02	2.5
	Right Hand	nvd	nvd	nvd	Right Hand	33	1.09E+02	2.0

APPENDIX T: A: number of cDNA copies per sandwich recovered from each half of a sandwich prepared by food handlers contaminated with norovirus GI gloved hands B: number of cDNA copies per sandwich recovered from each half of a sandwich prepared by food handlers contaminated with norovirus GI gloved hands

A.

Exp Number	Sample Name	cDNA copies per µl of RNA EXTRACT	Average cDNA copies per RNA EXTRACT	% recovery	Exp Number	Sample Name	cDNA copies per µl of RNA EXTRACT	Average cDNA copies per RNA EXTRACT	% recovery
Exp 1	Sandwich	24,964	24,207	0.56%	Exp 1	Sandwich	23,449	10,045	0.23%
	Sandwich	12,811				Sandwich	7,280		
Exp 3	Sandwich	2,648	3,169	0.07%	Exp 3	Sandwich	3,690	770	0.02%
	Sandwich	947				Sandwich	593		
Exp5	Sandwich	346	24,954	0.58%	Exp5	Sandwich	49,562	12,681	0.30%
	Sandwich	14,993				Sandwich	10,369		

B.

Exp Number	Sample Name	cDNA copies per µl of RNA EXTRACT	Average cDNA copies per RNA EXTRACT	% recovery	Exp Number	Sample Name	cDNA copies per µl of RNA EXTRACT	Average cDNA copies per RNA EXTRACT	% recovery
Exp 1	Sandwich	nvd[1]			Exp 1	Sandwich	9	7	0.01%
	Sandwich	5				Sandwich	5		
Exp 3	Sandwich	nvd			Exp 3	Sandwich	32	114	0.16%
	Sandwich	1				Sandwich	195		
Exp5	Sandwich	37	124	0.17%	Exp5	Sandwich	83	122	0.17%
	Sandwich	211				Sandwich	161		

[1]nvd=no virus detected

APPENDIX U: norovirus negative control experiments 1 & 2 of norovirus GI simulations and internal control virus Cq values

Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	28.5			
Negative inoculum	nvd	32.2	Negative inoculum	nvd	32.2
Negative inoculum	nvd	32.3	Negative inoculum	nvd	32.3
Food handler 1 Left hand	nvd	33.2	Food handler 1 Left hand	nvd	33.2
Food handler 1 Right hand	nvd	31.8	Food handler 1 Right hand	nvd	31.5
Food handler 2 Left hand	nvd	32.2	Food handler 2 Left hand	nvd	32.4
Food handler 2 Right hand	nvd	32.8	Food handler 2 Right hand	nvd	31.7
Food handler 3 Left hand	nvd	32.8	Food handler 3 Left hand	nvd	33.1
Food handler 3 Right hand	nvd	32.6	Food handler 3 Right hand	nvd	35.8
Sandwich	nvd	34.5	Sandwich	nvd	32.9
Sandwich	nvd	32.8	Sandwich	nvd	34.6
Preparation tray	nvd	34.9	Preparation tray	nvd	33.2
Preparation tray	nvd	34.6	Preparation tray	nvd	33.5
Lettuce bowl	nvd	34.0	Lettuce bowl	nvd	33.1
Lettuce bowl	nvd	34.8	Lettuce bowl	nvd	32.5
Sandwich tray	nvd	34.1	Sandwich tray	nvd	32.4
Sandwich tray	nvd	33.1	Sandwich tray	nvd	32.2
Negative beads	nvd	31.3			

[1]nvd=no virus detected

APPENDIX U: norovirus negative control experiments 3 & 4 GI simulations and internal control virus Ct values

Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	32			
Negative inoculum	nvd	34.4	Negative inoculum	nvd	35.2
Negative inoculum	nvd	35.1	Negative inoculum	nvd	36
Food handler 1 Left hand	nvd	34.3	Food handler 1 Left hand	nvd	35
Food handler 1 Right hand	nvd	34.9	Food handler 1 Right hand	nvd	35.1
Food handler 2 Left hand	nvd	34.1	Food handler 2 Left hand	nvd	35
Food handler 2 Right hand	nvd	34.4	Food handler 2 Right hand	nvd	36.8
Food handler 3 Left hand	nvd	35.8	Food handler 3 Left hand	nvd	34.9
Food handler 3 Right hand	nvd	35.6	Food handler 3 Right hand	nvd	35.9
Sandwich	nvd	35.3	Sandwich	nvd	35.1
Sandwich	nvd	35.1	Sandwich	nvd	38.5
Preparation tray	nvd	36	Preparation tray	nvd	36.1
Preparation tray	nvd	36.5	Preparation tray	nvd	35.9
Lettuce bowl	nvd	36.1	Lettuce bowl	nvd	35.6
Lettuce bowl	nvd	37.1	Lettuce bowl	nvd	35.5
Sandwich tray	nvd	36.4	Sandwich tray	nvd	35.7
Sandwich tray	nvd	36.2	Sandwich tray	nvd	38.1
Negative beads	nvd	38.4			

[1]nvd=no virus detected and internal control virus Ct values

APPENDIX U: norovirus negative control experiments 5 & 6 GI simulations and internal control virus Ct values

Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	34.1			
Negative inoculum	nvd	nvd	Negative inoculum	nvd	34.1
Negative inoculum	nvd	33.7	Negative inoculum	nvd	32.7
Food handler 1 Left hand	nvd	32.8	Food handler 1 Left hand	nvd	32.3
Food handler 1 Right hand	nvd	33.6	Food handler 1 Right hand	nvd	31.6
Food handler 2 Left hand	nvd	33.5	Food handler 2 Left hand	nvd	33.4
Food handler 2 Right hand	nvd	33.1	Food handler 2 Right hand	nvd	32.0
Food handler 3 Left hand	nvd	33.3	Food handler 3 Left hand	nvd	31.7
Food handler 3 Right hand	nvd	32.3	Food handler 3 Right hand	nvd	32.0
Sandwich	nvd	31.3	Sandwich	nvd	35.3
Sandwich	nvd	34.4	Sandwich	nvd	33.3
Preparation tray	nvd	32.7	Preparation tray	nvd	35.3
Preparation tray	nvd	32.5	Preparation tray	nvd	34.6
Lettuce bowl	nvd	33.6	Lettuce bowl	nvd	35.0
Lettuce bowl	nvd	33.1	Lettuce bowl	nvd	33.2
Sandwich tray	nvd	32.7	Sandwich tray	nvd	35.3
Sandwich tray	nvd	32.5	Sandwich tray	nvd	34.6
Negative beads	nvd	28.7			

[1]nvd=no virus detected

APPENDIX V: norovirus negative control experiments 1 & 2 of norovirus GII simulations and internal control virus Cq values

Sample name	Norovirus genogroup II	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup II	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	29.8			
Negative inoculum	nvd	32.6	Negative inoculum	nvd	32.4
Negative inoculum	nvd	33.1	Negative inoculum	nvd	33.1
Food handler 1 Left hand	nvd	34.2	Food handler 1 Left hand	nvd	31.9
Food handler 1 Right hand	nvd	35.2	Food handler 1 Right hand	nvd	34.5
Food handler 2 Left hand	nvd	35.1	Food handler 2 Left hand	nvd	34.7
Food handler 2 Right hand	nvd	32.6	Food handler 2 Right hand	nvd	32.2
Food handler 3 Left hand	nvd	32.2	Food handler 3 Left hand	nvd	33.9
Food handler 3 Right hand	nvd	33.0	Food handler 3 Right hand	nvd	33.5
Sandwich	nvd	31.7	Sandwich	nvd	36.2
Sandwich	nvd	33.1	Sandwich	nvd	34.7
Preparation tray	nvd	31.8	Preparation tray	nvd	33.7
Preparation tray	nvd	33.0	Preparation tray	nvd	34.5
Lettuce bowl	nvd	32.8	Lettuce bowl	nvd	33.2
Lettuce bowl	nvd	33.3	Lettuce bowl	nvd	34.6
Sandwich tray	nvd	33.7	Sandwich tray	nvd	33.9
Sandwich tray	nvd	33.1	Sandwich tray	nvd	33.1
Negative beads	nvd	20.5			

[1]nvd=no virus detected

APPENDIX V: norovirus negative control experiments 3 & 4 of norovirus GII simulations and internal control virus Cq values

Sample name	Norovirus genogroup II	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup II	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	30.8			
Negative inoculum	nvd	32.0	Negative inoculum	nvd	32.0
Negative inoculum	nvd	32.0	Negative inoculum	nvd	32.0
Food handler 1 Left hand	nvd	32.0	Food handler 1 Left hand	nvd	32.4
Food handler 1 Right hand	nvd	32.5	Food handler 1 Right hand	nvd	31.3
Food handler 2 Left hand	nvd	31.6	Food handler 2 Left hand	nvd	33.1
Food handler 2 Right hand	nvd	31.8	Food handler 2 Right hand	nvd	32.9
Food handler 3 Left hand	nvd	32.3	Food handler 3 Left hand	nvd	32.1
Food handler 3 Right hand	nvd	31.9	Food handler 3 Right hand	nvd	32.2
Sandwich	nvd	33.2	Sandwich	nvd	31.4
Sandwich	nvd	33.0	Sandwich	nvd	32.6
Preparation tray	nvd	32.0	Preparation tray	nvd	32.1
Preparation tray	nvd	32.3	Preparation tray	nvd	34.6
Lettuce bowl	nvd	31.5	Lettuce bowl	nvd	32.0
Lettuce bowl	nvd	32.2	Lettuce bowl	nvd	33.0
Sandwich tray	nvd	32.8	Sandwich tray	nvd	32.2
Sandwich tray	nvd	32.8	Sandwich tray	nvd	34.6
Negative beads	nvd	33.6			

[1]nvd=no virus detected

APPENDIX V: norovirus negative control experiments 5 & 6 of norovirus GII simulations and internal control virus Ct values

Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)	Sample name	Norovirus genogroup I	Mengovirus Internal control (Ct)
Mengo 10-5 QC	nvd[1]	28.1			
Negative inoculum	nvd	33.5	Negative inoculum	nvd	33.5
Negative inoculum	nvd	33.1	Negative inoculum	nvd	33.1
Food handler 1 Left hand	nvd	30.7	Food handler 1 Left hand	nvd	29.9
Food handler 1 Right hand	nvd	32.5	Food handler 1 Right hand	nvd	31.5
Food handler 2 Left hand	nvd	32.5	Food handler 2 Left hand	nvd	31.0
Food handler 2 Right hand	nvd	32.5	Food handler 2 Right hand	nvd	31.6
Food handler 3 Left hand	nvd	31.4	Food handler 3 Left hand	nvd	31.1
Food handler 3 Right hand	nvd	32.2	Food handler 3 Right hand	nvd	30.9
Sandwich	nvd	31.1	Sandwich	nvd	32.5
Sandwich	nvd	31.7	Sandwich	nvd	33.2
Preparation tray	nvd	32.2	Preparation tray	nvd	32.2
Preparation tray	nvd	30.3	Preparation tray	nvd	32.1
Lettuce bowl	nvd	31.6	Lettuce bowl	nvd	31.5
Lettuce bowl	nvd	32.0	Lettuce bowl	nvd	33.2
Sandwich tray	nvd	32.6	Sandwich tray	nvd	32.6
Sandwich tray	nvd	32.4	Sandwich tray	nvd	33.0
Negative beads	nvd	33.4			

[1]nvd=no virus detected

APPENDIX W: F-test for A. norovirus GI and B. norovirus GII simulations to determine if variance are equal

A.F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	4.430366	4.135682
Variance	3.003714	2.95651
Observations	17	17
df	16	16
F	1.015966	
P(F<=f) one-tail	0.487559	
F Critical one-tail	2.333484	

B.F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	3.151043	2.35138
Variance	1.234262	1.616763
Observations	13	16
df	12	15
F	0.763415	
P(F<=f) one-tail	0.322683	
F Critical one-tail	0.382139	

APPENDIX X: Student t test for A. norovirus GI and B. norovirus GII simulations

A.t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	4.430366	4.135682
Variance	3.003714	2.95651
Observations	17	17
Pooled Variance	2.980112	
Hypothesized Mean Difference	0	
df	32	
t Stat	0.497679	
P(T<=t) one-tail	0.311056	
t Critical one-tail	1.693889	
P(T<=t) two-tail	0.622113	
t Critical two-tail	2.036933	

B.t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	3.151043	2.35138
Variance	1.234262	1.616763
Observations	13	16
Hypothesized Mean Difference	0	
df	27	
t Stat	1.806296	
P(T<=t) one-tail	0.041016	
t Critical one-tail	1.703288	
P(T<=t) two-tail	0.082032	
t Critical two-tail	2.051831	